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[*continued on p. iii of cover*

The Effect of High Oxygen Pressure on the Respiratory Metabolism of Pea Seeds

G. G. PRITCHARD

The Botany School, University of Cambridge

WITH FOUR FIGURES IN THE TEXT

Received 10 February 1961

SUMMARY

The time course of the changes in concentration of certain of the intermediates of the Krebs cycle (and derivatives of these intermediates) in the mature pea seed during the inhibition of respiration by high oxygen pressure has been studied. The results support the conclusion that the primary effect of high oxygen pressure is an inhibition of the oxidation of citric acid by the Krebs cycle. Aconitase appears to be the most likely site of inhibition. In the later stages of treatment there appears to be a slowing of the rate of glycolysis either as a consequence of the primary inhibition or due to direct inhibition of other enzymes. The highest rate of citrate accumulation observed in this work would allow 50 per cent. of the aerobic carbon dioxide output of the pea seed to be derived from the Krebs cycle.

INTRODUCTION

TURNER and Quartley (1956, 1957), studying the effects of high oxygen pressure on the respiration of mature pea seeds, concluded that the Krebs cycle was a major pathway in the respiration of peas. Wager (1961) has made a very careful study of the concentration changes in the Krebs cycle acids in peas during and after a period of anaerobiosis, from which he concludes that the cycle can be operating only sluggishly. Therefore a reinvestigation of the effects of high oxygen pressure on the respiration and Krebs cycle acid concentrations in 'Onward' peas (the variety used by Wager) has been carried out. An improved technique for the measurement of carbon dioxide output under high oxygen pressure conditions (Barker, 1960) has been used. The analytical methods are essentially the same as those used by Wager.

The work of Barker and Mapson (1955) showed that high oxygen pressure interferes with the metabolism of citric acid in the potato tuber and from changes in concentration of citrate and of other acids a block in the Krebs cycle was inferred. This work was confirmed by Turner and Quartley (1956) using peas. In the present study the determination of a wider range of compounds and a closer study of the sequence of changes has yielded further information on the effects of high oxygen pressure on respiratory metabolism.

EXPERIMENTAL METHODS

The apparatus used for holding peas at high oxygen pressures has been described by Barker (1960). Peas were shelled immediately after harvesting

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EXPERIMENTAL METHODS

The apparatus used for holding peas at high oxygen pressures has been described by Barker (1960). Peas were shelled immediately after harvesting

and samples were placed either in large glass vessels for use as 'air controls' or in metal pressure vessels. A current of CO_2-free air at 2 litres per hour was passed over both series of samples for about 18 hours after harvesting. Then in the pressure vessels the current of air was replaced by a current of pure oxygen and the pressure raised to the desired level. By increasing the rate of flow of oxygen through the vessel by a factor equal to the number of atmospheres pressure applied (Barker, 1960) the initial drop in the apparent carbon dioxide output found in the work of Caldwell (1956) and of Turner and Quartley (1956) is avoided. Thus a true record of the carbon dioxide output from the tissue is obtained immediately after the application of pressure. Only a small portion of the space inside the pressure vessels is occupied by the peas and the remaining 'dead space volume' introduces slight discrepancies between the true and observed carbon dioxide output particularly during the first few hours after the tissue is placed in the vessel before pressure is applied. This is apparent in the experimental curves showing carbon dioxide output in Figs. 1–3 (the initial observed rate of carbon dioxide output from the pressure vessels being lower than that from the air controls). There is also a slight discrepancy between the true and apparent rates of carbon dioxide output during the inhibition following application of pressure. Calculations of the magnitude of this discrepancy over the first 24 hours show that it would not affect the observed values by more than 2 or 3 per cent.

No determinations have been made of the changes in dissolved and bound carbon dioxide during the course of the experiments. The decrease in pH of the cell sap during the inhibition by high oxygen pressure may result in a release of bound carbon dioxide from the tissue. Using Wager's data (1961) for total carbon dioxide content of peas and the data of Turner and Quartley (1956) for changes in pH during inhibition of respiration by high oxygen pressure it is possible to calculate the amount of carbon dioxide that would be released. It cannot be assumed that the pH measurements represent the true pH in the cells but even taking changes twice as great as those measured the release of bound carbon dioxide during the inhibition would only contribute 3 per cent. of the total carbon dioxide output over the first 24 hours.

The experiments were conducted in a constant temperature darkroom at 15° C.

Keto-acid concentrations were determined by the method of Isherwood and Cruickshank (1954) employing certain modifications introduced by Niavis (1956) particularly the cold killing technique of Isherwood and Niavis (1956). This yields reproducible values and minimizes killing artefacts.

Stable carboxylic acids present in concentrations greater than 3 mg. per 100 g. fresh weight of tissue were determined by the silica gel column chromatographic method of Wager and Isherwood (unpublished). The technique yields quantitative recoveries of pure acids and reproducible results from tissue extracts.

The pentabromacetone method of Hargreaves, Abrahams, and Vickery (1951), which employs a trichloroacetic acid extraction technique, was used for

determining citric acid concentration. It gave values about 25 per cent. higher than those determined by the column technique. This discrepancy was traced very largely to incomplete extraction of the citric acid from the tissue by 70 per cent. alcohol—the extraction medium used in the column technique. Six extractions with 70 per cent. alcohol were carried out over the course of 24 hours and separate determinations on these successive extractions showed that

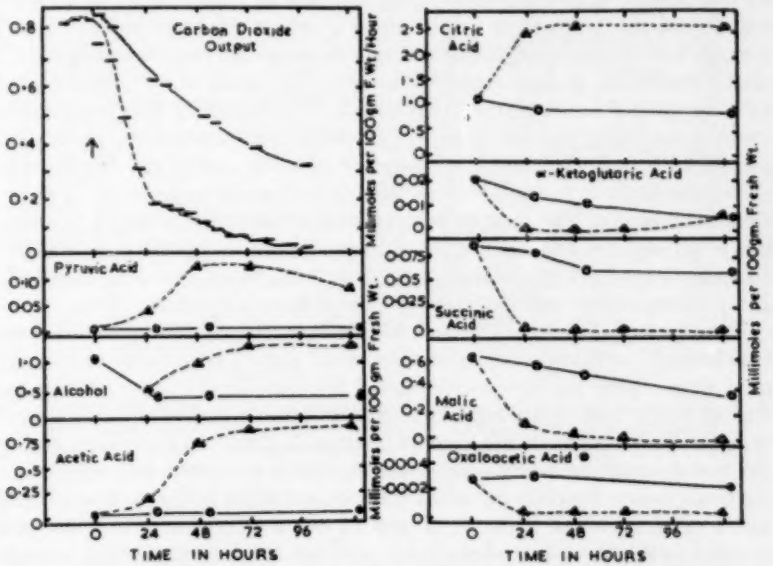

FIG. 1. *Experiment I.* The changes at 15° C. in the rate of carbon dioxide output and organic acid concentrations of samples of 'Onward' pea seeds maintained in air (continuous line, circles) or subjected to oxygen at 3½ atmospheres pressure (broken line, triangles).

while the bulk of the citrate was removed in the first two extractions, small quantities of citrate were still extractable after six extractions. It may be that some of the citrate was present as the sparingly soluble calcium salt. Extraction of the residue with weak sulphuric acid and chromatographic analysis of this extract showed that citric acid was the only acid incompletely extracted by the alcohol. Accordingly the pentabromacetone values for citric acid have been used as a more accurate estimate of the total citrate content of the tissue and it is these values that are shown in Figs. 1–4.

Alcohol was determined by steam distillation from a metaphosphoric acid extract of the tissue and subsequent oxidation and back titration followed the procedure of Friedmann and Klaas (1936).

RESULTS

Experiment I. Fig. 1 shows the effects of a 5-day sojourn in 3½ atmospheres of pure oxygen on the rate of carbon dioxide output and concentration of

organic acids. Samples were taken at 24-hour intervals. The peas for this experiment were obtained from Histon, Cambridge, on 9 July 1957.

The rate of carbon dioxide production falls in air after harvesting the peas. High oxygen pressure induces a rapid inhibition of carbon dioxide output which continues for about 24 hours, after which the rate falls more slowly.

The application of high oxygen pressure brings about a marked increase in the concentration of citric acid during the first 24 hours. After 24 hours it remains relatively steady at a level about 2½ times the concentration in air. Pyruvic acid, alcohol, and acetic acid also show an increase in concentration under conditions of high oxygen pressure. The cause of the initially high value for alcohol concentration is not known. The increase in these compounds commences during the first 24 hours; the most rapid increase occurs between 24 and 48 hours. Compounds subsequent to citric acid in the Krebs cycle show a decrease to a very low concentration in the first 24 hours at 3½ atmospheres of oxygen. The concentration of α-ketoglutarate, after falling to a very low level, starts to rise again after 48 hours. Other acid peaks detectable by column chromatography (Wager, 1961; Pritchard, 1959) show little change in size or composition under conditions of high oxygen pressure.

Experiment II (Fig. 2). The peas for this experiment were obtained from Gamlingay, Cambridgeshire, on 24 September 1957. The experimental conditions were exactly the same as for Expt. I but samples were taken during the first 24 hours. The air drift and high oxygen pressure inhibition of the rate of carbon dioxide output is the same as in Expt. I. Citric acid increases during the first 5 hours. Similarly α-ketoglutarate shows a considerable decrease in the first 5 hours. Succinic and malic acids show a slight fall in the first 5 hours and a more rapid fall between 11 and 24 hours. Oxaloacetic acid shows a decrease in the first 5 hours (see, however, Expt. III below). The time-course of the concentration changes in acetic and pyruvic acids is of interest (alcohol concentration was not determined in this experiment). The concentration of these acids remains the same as in air during the first 11 hours in oxygen and then starts to rise between 11 and 24 hours.

Experiment III (Fig. 3). The aims of this experiment were, first, to confirm the sequence of changes found in Expt. II and, secondly, to compare the rate of change in carbon dioxide output and organic acid concentration at 3½ atmospheres oxygen with those at 10 atmospheres. This experiment was carried out twice. The first attempt gave very variable carbon dioxide output values probably owing to leaks in the pressure vessels. The graphs in Fig. 3 are from the second attempt except for the keto-acid curves, which are from the first of the two experiments.

'Onward' peas were obtained from Guilden Morden, Cambridgeshire, on 5 August 1958. The experimental conditions were the same as in the first two experiments. Even at a pressure of 10 atmospheres there was no significant decrease in the rate of carbon dioxide output in the first 3 hours. After this the rate was inhibited much more rapidly at 10 atmospheres than at 3½ atmospheres. The concentration reached by citric acid after 24 hours is about the

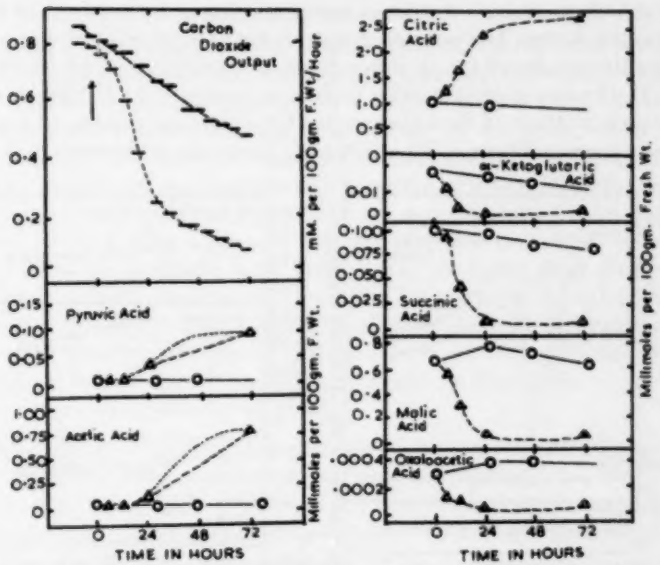

FIG. 2. *Experiment II.* The changes at 15° C. in the rate of carbon dioxide output and organic acid concentrations of samples of 'Onward' pea seeds maintained in air (continuous line, circles) or subjected to oxygen at 3½ atmospheres pressure (broken line, triangles) showing the sequence of changes during the first 24 hours of treatment.

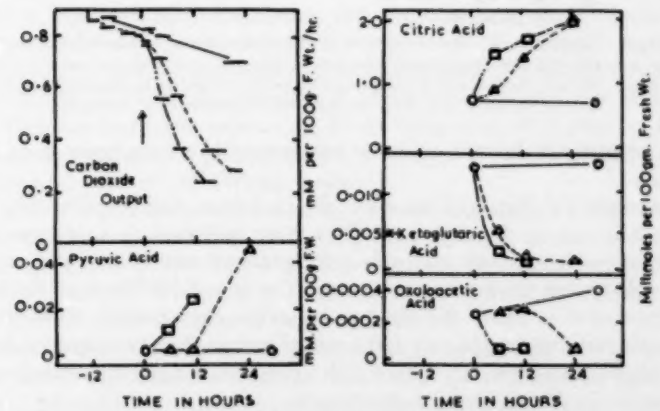

FIG. 3. *Experiment III.* The changes at 15° C. in the rate of carbon dioxide output and organic acid concentrations of samples of 'Onward' pea seeds maintained in air (continuous line, circles) or subjected to oxygen at 3½ atmospheres pressure (broken line, triangles) or at 10 atmospheres pressure (line interrupted by dots, squares).

same at both pressures—the initial rate of increase is, however, much greater at 10 atmospheres. The rates of change in the concentration of Krebs cycle intermediates induced by 3½ atmospheres compare very well with those in Expt. II, the only exception being in the time course of the decrease in oxaloacetic acid. In Expt. II the concentration fell in the first 5 hours. In Expt. III the concentration does not fall significantly below the air concentration in the

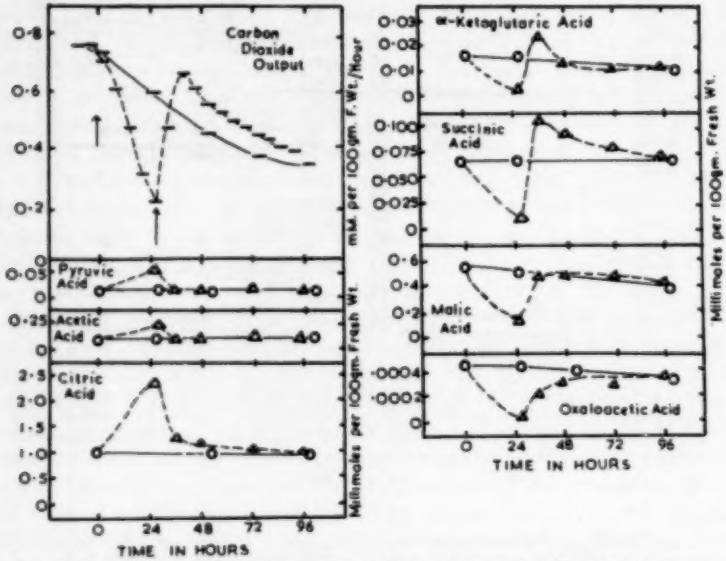

FIG. 4. *Experiment IV*. The changes at 15° C. in the rate of carbon dioxide output and organic acid concentrations of samples of 'Onward' pea seeds maintained in air (continuous line, circles) and of samples subjected to oxygen at 3½ atmospheres pressure for 24 hours and then returned to air (broken line, triangles).

first 11 hours. Oxaloacetic acid was not detectable after 5 hours at 10 atmospheres.

Experiment IV (Fig. 4). Peas were obtained from Guilden Morden, Cambridgeshire, on 19 August 1957. They were subjected to a pressure of 3½ atmospheres for 24 hours, after which the pressure was released and a current of air substituted for the oxygen stream. The rate of inhibition of the carbon dioxide output is much the same as in earlier experiments. Following the release of pressure a rapid rise in the rate of carbon dioxide output occurs to a value above the rate in air. It then falls again but remains significantly above the rate in air throughout the experiment.

Following release of pressure the concentration of all the acids returns rapidly or more gradually to the air-concentration levels, α-ketoglutarate and succinate increasing initially above these levels. Thus the tissue is not irreversibly damaged during the 24-hour sojourn in 3½ atmospheres pure oxygen.

DISCUSSION

The most obvious interpretation of the present data is that of Turner and Quartley (1956) and Barker and Mapson (1955), namely, that citrate increases in concentration because its oxidation by the Krebs cycle is inhibited by high oxygen pressure. Citrate continues to be formed by the action of the condensing enzyme and therefore increases in concentration. α-Ketoglutarate, succinate, malate and oxaloacetate which are the products of further metabolism of citrate in the Krebs cycle decrease in concentration. The enzyme inhibited by high oxygen pressure is either aconitase or isocitric dehydrogenase. Aconitase seems the more likely site of inhibition since there is no detectable increase in isocitrate concentration. An increase in the concentration of isocitrate would have been detected by silica-gel column chromatography. As isocitrate is not present in detectable quantities in peas kept in air (Pritchard, 1959, cf. Wager, 1961) it would not be possible to demonstrate a decrease.

Two further questions may be asked:

1. What effects of high oxygen pressure on respiratory metabolism other than the primary effect on citrate oxidation are indicated?

2. Can the data give an estimate of the contribution made by the Krebs cycle to the total carbon dioxide output in peas under these conditions?

1. The concentration of citric acid rises fairly steadily during the first 24 hours at high oxygen pressure, after which time it increases much more slowly. This change in rate is probably due to a slower rate of synthesis, which could be attributed to a direct inhibition of the condensing enzyme or to a limiting supply of acetyl CoA or of oxaloacetate. Of these possibilities the limiting concentration of oxaloacetate is the one best supported by the data. It can be seen from Figs. 1–3 that oxaloacetate concentration has indeed dropped to an undetectable value by 24 hours and the compounds maintaining a supply of oxaloacetate, viz. malate, succinate and α-ketoglutarate, are virtually depleted after 24 hours. The slow accumulation of citrate after 24 hours may be due to a very slow residual traffic around the Krebs cycle or to alternative routes of oxaloacetate synthesis, e.g. carboxylative formation of oxaloacetate from phospho-enol pyruvic acid (Wager, unpubl.).

Sources of oxaloacetate other than reserves of Krebs cycle acids must be postulated, since citrate accumulation is greater than the measured decrease of α-ketoglutarate, malate, and succinate, especially during the period 11–24 hours (see Table I). Oxidation of amino-acids, e.g. glutamate and aspartate, would provide another source of oxaloacetic acid.

When the concentration of oxaloacetate drops to such an extent that the rate of citrate synthesis slows down, then acetyl CoA formed by glycolysis must be diverted into alternative pathways. Such a diversion (to acetoacetate) was demonstrated by Recknagel and Potter (1951) in liver. They inhibited the Krebs cycle by malonate, thereby preventing regeneration of oxaloacetate. In the present case the acetic acid which accumulates may be derived from acetyl CoA, though other routes of acetic acid synthesis are known. Work in

progress in which ^{14}C-labelled glucose was fed to carrot disks has shown the accumulation of a large amount of radioactivity in an unidentified compound under high oxygen pressure conditions. This compound has a very high Rf value in most solvents and may be another product of acetyl CoA metabolism.

TABLE I

Balance of losses and gains within the Krebs cycle (from the results of Experiment II). All figures are in millimoles per 100 g. fresh weight

Time interval	Citric acid accumulated	α-Ketoglutaric ac. loss	Succinic ac. loss	Malic ac. loss	Total losses	Deficit
0–5 hrs.	0·23	0·006	0·008	0·10	0·114	0·116
5–11 hrs.	0·36	0·007	0·053	0·24	0·30	0·06
11–24 hrs.	0·78	0·004	0·041	0·24	0·285	0·495
24–72 hrs.	0·40	—	—	0·06	0·06	0·34

TABLE II

Comparison of the inhibition of CO_2 output with the 'CO_2 equivalent' of the accumulated compounds. All figures are in millimoles per 100 g. fresh weight

Time interval	(1) CO_2 output in air	(2) CO_2 output in oxygen	(3) Difference	(4) Amount of CO_2 derivable from accumulated compounds.	(5) CO_2 output in oxygen plus CO_2 equivalent of accumulated compounds.
Experiment I					
0–24 hrs.	18·20	13·32	4·88	3·05	16·37
24–48 hrs.	13·32	3·66	9·66	2·70	6·36
48–72 hrs.	8·58	1·42	7·16	1·12	2·54
Experiment II					
0–5 hrs.	4·82	4·53	0·29	0·46	4·99
5–11 hrs.	4·72	4·20	0·52	0·72	4·92
11–24 hrs.	8·57	5·94	2·63	1·79	7·73
0–24 hrs.	18·11	14·46	3·44	2·97	17·46
24–72 hrs.	26·02	6·84	19·18	4·23	11·07
Experiment III					
3½ atmospheres					
0–24 hrs.	17·73	14·06	3·67	2·81	16·87
10 atmospheres					
0–5 hrs.	4·83	3·87	0·96	1·36	5·23

That compounds other than those measured in this study must be accumulating is suggested by comparing the amount by which carbon dioxide production is inhibited with the calculated amount of carbon dioxide which would have been produced if the accumulated intermediates had been oxidized by the Krebs cycle. This comparison is shown in Table II. The figures in column 4 for the amount of carbon dioxide produced by oxidation of citrate, acetate, alcohol and pyruvate are derived by assuming that oxidation of a mole of alcohol, acetate or citrate would give two moles of carbon dioxide and oxidation of a mole of pyruvate three moles of carbon dioxide.

In the first 24 hours, accumulation of pyruvate, alcohol, acetate, and citrate accounts for from 62–86 per cent. of the inhibition of carbon dioxide output. After 24 hours only 22 per cent. of the inhibition is accounted for by accumulation of these compounds. This unaccounted-for inhibition must be attributed either to a decrease in the rate of glycolysis (or other respiratory pathways) or to an accumulation of compounds not determined in the present study.

The accumulation of pyruvate and alcohol may be a consequence of the reduced rate of regeneration of free CoA (due to the accumulation of acetyl CoA) or to a separate inhibition of the pyruvic oxidase system as postulated by Dickens (1946). The rate of pyruvate, alcohol, and acetate accumulation decreases after 48 hours, probably indicating an inhibition of glycolysis either by direct enzymic inhibition or due to a reduced rate of ATP formation.

2. It is difficult to make an assessment from the data of the contribution which the Krebs cycle makes to the total carbon dioxide output. The chief difficulty is that the pea seed is a complex tissue; the compounds determined are probably not distributed homogeneously throughout the seed and probably not homogeneously within individual cells. Granted certain assumptions the rate of citrate accumulation could be used to provide an overall estimate for the whole seed.

If it is assumed that the rate of glycolysis is not affected by high oxygen pressure and that the block at aconitase is complete then the maximal rate of citrate synthesis will give a measure of the rate at which citrate is being synthesized by the condensing enzyme.

If glycolysis rate were altered by high oxygen pressure treatment it would be expected that the 'carbon dioxide equivalent' of the accumulated compounds (i.e. the quantity shown in column 4 of Table II) plus the carbon dioxide output in oxygen would differ from the carbon dioxide output in air. This sum is shown in column 5 of Table II. It slightly exceeds the carbon dioxide output in air in the first 11 hours and is slightly less over the period 11–24 hours. The excess in the first 11 hours (3 per cent.) is of the order of the calculated release of bound carbon dioxide. Thus there is evidently no very large increase or decrease in the rate of glycolysis over the first 24 hours (cf., however, the situation in apples, Barker *et al.*, 1960).

The assumption that the block at aconitase is complete is less justifiable. It is evident from Expt. III that an increase in pressure from $3\frac{1}{2}$ atmospheres to 10 atmospheres results in a higher rate of citrate accumulation, which implies that the block is only partial at $3\frac{1}{2}$ atmospheres. If the block were only partial then the rate of oxaloacetate production should not be such as to limit citrate synthesis after 24 hours. However, this apparent difficulty only arises if it is assumed that the inhibition of aconitase is rapid and reaches a steady level after a short period. It is more probable that the inhibition of the enzyme is a gradual process and increases progressively over the 24-hour period and perhaps after that. For about the first 12 hours at $3\frac{1}{2}$ atmospheres pressure the rate of citrate accumulation is increasing owing to progressive increase in the inhibition. Between 12 and 24 hours the decreasing concentration of oxalo-

acetate reduces this rate of increase, until after 24 hours it is so low that citrate accumulation almost ceases. Thus the form of the citrate-increase curve should be more nearly sigmoid than the experimental curves based on a few sampling points indicate. At 10 atmospheres a more rapid inhibition of aconitase results in a higher initial rate of citrate accumulation. The consequent more rapid decline in oxaloacetate concentration results after 12 hours in a much slower rate of citrate synthesis (see Fig. 3).

This gradual inhibition of citrate oxidation means that the observed maximal rate of citrate accumulation is not necessarily the maximal rate of citrate synthesis by the condensing enzyme. The maximal rates observed are: 0·06 mM. per hour at 3½ atmospheres and 0·136 mM. per hour at 10 atmospheres. A rate of 0·136 mM. per hour would, if the citrate were completely oxidized by the Krebs cycle, give a carbon dioxide output of 0·41 mM. per hour, i.e. about 50 per cent. of the carbon dioxide output in air. This estimate agrees with the maximum estimates given by Gibbs and Beevers (1955) for pea stems and leaves and with the *in vitro* studies of Mapson and Moustafa (1956), who found that 50 per cent. of the oxygen uptake of the germinating pea was mitochondrial in origin.

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Growth Substances in the Roots of *Vicia faba*

II. THE EFFECTS OF AGEING AND EXCISION OF THE MAIN TAP-ROOT MERISTEM[1]

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WITH SIX FIGURES IN THE TEXT

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SUMMARY

A quantitative chromatographic study has been made of the changes in the activity and distribution of the main ether-soluble acid auxins of *Vicia faba* seedling root systems during development and resulting from excision of the main tap-root meristem.

An IAA-like auxin (AP (*ii*)) is apparently synthesized predominantly in apical and at a lower rate in lateral meristems. Production seems to stop when meristematic growth stops. Its concentration in mature extended cells is much lower and may fall to zero in old cells, suggesting active degradation by an auxin-oxidase. Excision of the main tap-root tip gradually results in a greatly augmented production of AP (*ii*) in lateral meristems, conceivably the *result* of correlative growth promotion.

A second auxin and root-growth inhibitor (AP (*iii*)) is present at higher activity levels than AP (*ii*) in tap-root meristems and at the same level in lateral meristems. In mature cells its activity is much lower than that of AP (*ii*). In contrast to AP (*ii*) it accumulates in both tap-root meristems and mature tissue as the root system ages. It could also be produced during meristematic growth but is not subsequently degraded. As with AP (*ii*), excision of the tap-root tip brings about a great increase in its concentration in lateral tips.

A third auxin and root-growth accelerator (AP (*i*)) (accelerator α?) is present in lower concentrations in both meristem and mature tissue. Its concentration tends to decrease with ageing and, in lateral meristems, is not affected by tap-root tip excision.

It is suggested that AP (*ii*) produced by the meristem is normally at suboptimal levels in the extending cells and may be the principal hormone controlling extension growth. AP (*iii*) accumulation may account for growth deceleration on ageing. The role of AP (*i*) remains obscure. It is unlikely that correlative effects of the tap-root tip on lateral root growth are exercised directly via these auxins.

INTRODUCTION

OBSERVATIONS of auxin gradients in growing organs and of the drifts in their concentrations with development have long been one of the most important ways of approaching the problem of auxin control of growth in the

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plant as a whole. Compared with shoot systems, root systems have been little studied, even though one might expect the general organization of growth correlations to be simpler in roots. Furthermore, almost all the related quantitative estimates of auxin concentration have been made with crude extracts or diffusates assayed by coleoptile or root curvature tests. Results from such methods have not always been consistent, probably because they cannot reveal changes which undoubtedly occur in the proportions of growth promoters and inhibitors present in such extracts. Many studies have been made of these several components in extracts of whole roots (for bibliography see Åberg, 1957; Pilet, 1958) by the method of paper partition chromatography, but the application of these methods to the study of auxin behaviour in relation to root development has only just begun. Thus Pilet (1958), using a root-tip assay, has investigated the changes in concentration of 'accelerator α', 'inhibitor β' (Bennet-Clark *et al.*, 1952), and an IAA-type auxin in passing from the tip to the base of seedling roots of *Lens culinaris*. The work to be described in the present paper has been concerned with the effects of ageing on the content of various growth substances in *Vicia faba* roots as determined by similar techniques. A study has been made of the growth substances of lateral roots also and the effects thereon of excision of the main tap-root tip.

METHODS

The methods employed for growing the *Vicia faba* seedlings, for extracting and separating the growth substance components by paper partition chromatography, and for assaying segments of the chromatogram have already been described in detail (Lahiri and Audus, 1960). Attention has so far been confined to the acid ether-soluble fraction of cold ethanol extracts and the chromatographic running solvent has been isobutanol-methanol-water. Assay has been by the *Avena* mesocotyl test of Nitsch (1956) which is very sensitive to auxins but, since the growth of control segments is small, does not detect inhibitors except in relatively high concentrations.

In *Vicia faba* roots three such acid-auxins can be consistently isolated in ethanol extracts: one designated AP (*i*) (Lahiri and Audus, 1960) running at Rf 0–0·25 has the properties of 'accelerator α' (Bennet-Clark *et al.*, 1952). Another, called AP (*ii*), running at Rf 0·35–0·65, close to indole-3-acetic acid (IAA), inhibits root growth but has properties suggesting that it is not IAA. The third, called AP (*iii*), inhibits root growth and runs at Rf 0·80–1·00. In addition to these three substances a fourth auxin sometimes appears on one-way chromatograms at Rf 0·30, but it is either a very weak auxin or is present in very low concentrations. Similarly the inhibitor-β complex has occasionally been detected at Rf 0·65–0·85. In these assays it is not possible to assess more than changes in activity which will reflect, but not be an exact measure of, concentration, since the nature of these substances is still unknown. Even with AP (*ii*), which most closely resembles IAA, it is not advisable to estimate its activity in terms of IAA-equivalents, since the activity-concentration curve of

the substance is quite different from that of IAA. In the results which follow, therefore, concentrations have been estimated on a purely arbitrary basis of activity by finding the areas of active spots on chromatograms, where responses fall above the 5 per cent. fiducial limits of the assay, and dividing these areas by the fresh weights of the tissue extracted for assay.

RESULTS

(*a*) *Drifts with age in the root system as a whole*

A series of preliminary observations was made using extracts of the whole root system of seedlings ranging in age from 3 to 25 days old. For each sample the total fresh weight of root per seedling was determined so that growth-rate curves could be calculated therefrom.

The results of these investigations are shown in Fig. 1. In young roots all three of the main auxins are present in relatively high concentrations. In addition, the fourth auxin, AP (*iv*) (Rf 0·30), appeared in the 3-day-old root and inhibitor β was also present. With the exception of AP (*ii*) the overall concentration of all these substances decreased as the root system increased in size and age. AP (*ii*), however, showed an initial increase, which is possibly significant, and reached a maximum at about the time when the root system was most actively growing. The most likely explanation of this behaviour is that AP (*ii*) arises in the meristem, probably as a concomitant of protein synthesis and possibly even linked with it via a common metabolite such as tryptophane. From the lack of any closely correlated changes in AP (*i*) or AP (*ii*) there is no suggestion in these data that these relatively long-term changes involve interconversions of the three auxins, as was suggested for AP (*ii*) and AP (*iii*) after geotropic stimulation (Audus and Lahiri, 1961). But if AP (*iii*) represents a relatively large bulk of a relatively inactive precursor of AP (*ii*), any small correlated changes in AP (*iii*) would be very difficult to detect with this degree of variability.

The rapid disappearance of AP (*iv*) and the somewhat slower loss of inhibitor β suggest that these may be substances from the seed which are either metabolized away or diluted down below the sensitivity of the assay in the increasing bulk of the root system as a whole. This is certainly not so for the three main auxins. As Fig. 2 shows, the total quantities of the three main auxins per root run very closely parallel to the increasing weight of the root and reach their maxima at about the time the root stops growing actively. This demonstrates that they are being continuously produced in the growing root, probably in the meristems (see later), and that this production stops when active meristematic growth stops. Furthermore, in the mature root some substances (e.g. AP (*i*) and AP (*ii*)) are actively metabolized away so that their concentrations rapidly fall. It is not impossible that this breakdown may also be going on in the actively growing root but that this is masked by the much more rapid production.

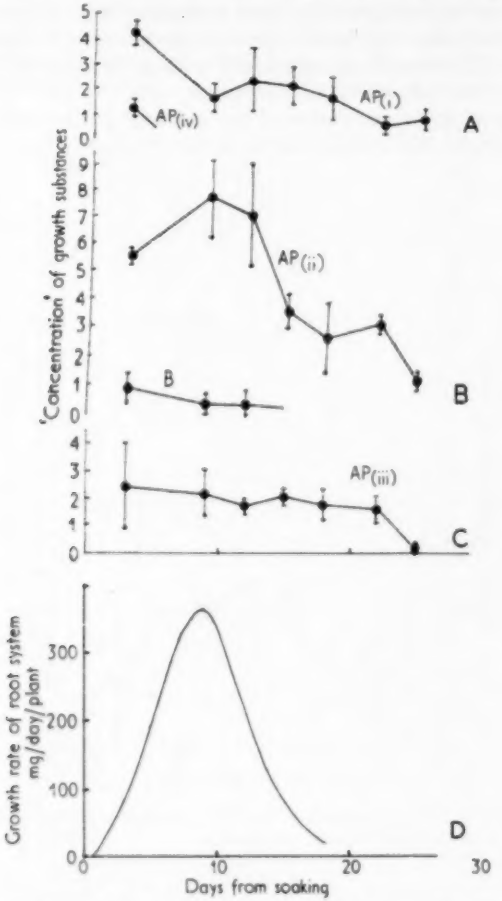

FIG. 1. Changes in growth-substance concentrations in the root system of *Vicia faba* seedlings in relation to growth and ageing.

A, B, and C. Growth-substance concentration drifts with age of root. The vertical lines are drawn at $\pm s$ on either side of the means of three observations. For further explanation see text.

D. Root system growth-rate curve.

(*b*) *Drifts with age in tap-root tips, mature root-bases and lateral roots*

The logical extension of these preliminary studies has been to make separate analyses of the tip of the main tap root (terminal 5 mm.), the emerged lateral roots and the mature tissues of the remaining tap-root bases. The 5-mm. tap-root tips comprise, in addition to the meristem, most of the cells of the extending region and time has not yet permitted a more detailed analysis of the different zones. Furthermore, no attempt was made to separate

lateral tips from the more mature basal regions of laterals. A further complication arises in that the basal region of tap roots will contain the non-emerged meristems of still younger laterals; these matters will be taken into consideration when the results are discussed.

The changes in the concentration of the various growth substances in these three components of the root system are shown in Figs. 3 to 5.

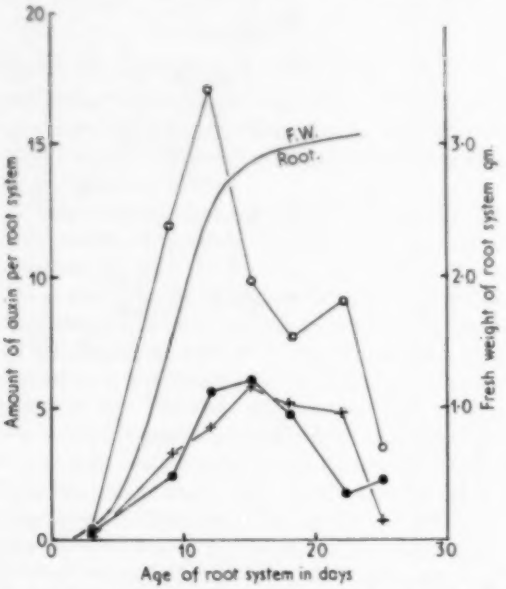

FIG. 2. Changes in the total amounts of the three principal acid auxins of *Vicia faba* roots during the first 25 days of growth after germination.

—●— AP (*i*) ('accelerator α')
—○— AP (*ii*)
—+— AP (*iii*)

Firstly, it is clear that the main tap-root tip (Fig. 3) contains a very much higher concentration of all three auxins than does either the basal region of the tap root (Fig. 4) or the lateral roots (Fig. 5). The ratios of concentrations for tap-root tip : tap-root base : lateral roots, are of the order of 30 : 1·5 : 1 for AP (*i*); 80 : 5 : 2 for AP (*ii*), and 130 : 3 : 2·5 for AP (*iii*). This is strong support for the suggestion, which arose from the preliminary experiments, that the tap-root tip is the main region of production of all three of these auxins and that their presence in the mature basal tissues is due to transport from the tip. The most surprising finding in this respect is the much lower concentrations of all three substances in the lateral roots, where they fall consistently below those in the main root base. AP (*i*) and AP (*ii*) are at lower levels even than in the root system taken as a whole (Fig. 1), while AP (*iii*) concentrations seem to be

about the same. In view of the fact that the lateral meristems have been included in the lateral tissue analysed, this would suggest that lateral-root tips contain very much less of these auxins than main tap-root tips, a point confirmed later by direct analysis. It must not be forgotten that the main root

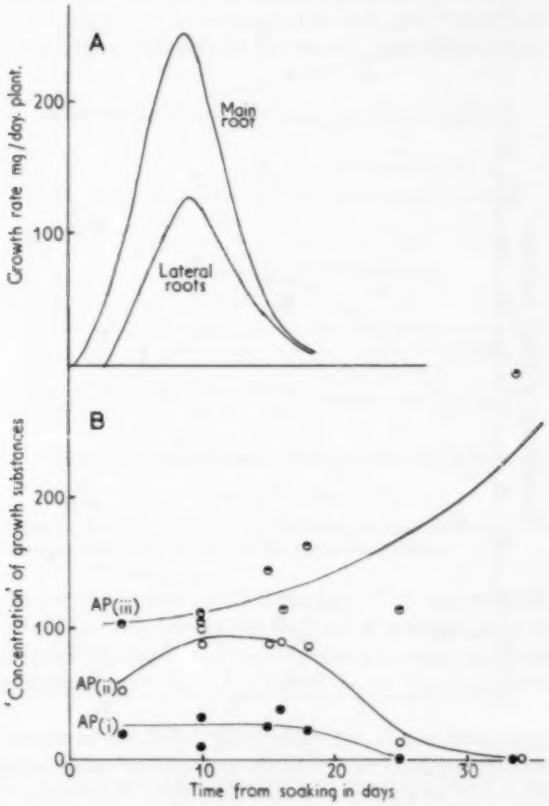

FIG. 3. Changes in the concentration of the three principal acid auxins in the 5-mm. tips of the main tap roots of *Vicia faba* in relation to growth-rate and ageing.

A. Growth-rate curves (increase in fresh weight) for main tap roots and for lateral roots.

B. Changes in auxin concentration.

bases contain small non-emerged lateral meristems, and if these possess relatively high concentrations of auxins the actual amounts of those substances in the mature tissue of the main root base may be somewhat lower than would appear from Fig. 4.

Turning first to the drifts with ageing in the main tap root, Figs. 3 and 4 show these effects in the 5 mm. tap-root tips and the bases without the laterals. The trend in AP (*ii*) in main tips (Fig. 3) is strikingly similar to that in

the root as a whole, showing a rise to a maximum a day or so after the time of maximum growth-rate in the main root. These concentrations fall away as root growth-rate decreases and this substance virtually disappears from root tips which have stopped growing (35 days). A very similar trend, although at

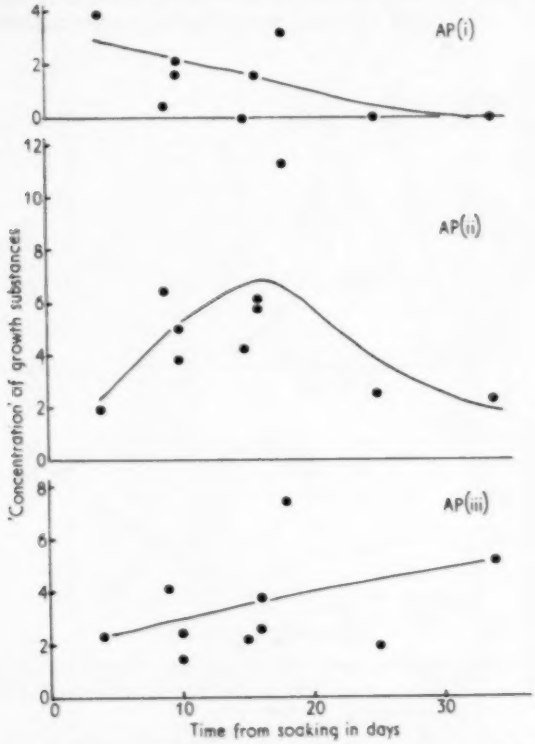

FIG. 4. Changes in the concentrations of the three principal acid auxins in the mature tissue of main tap roots of *Vicia faba* (minus main meristem and lateral roots) in relation to age of root system.

a much lower level and therefore showing much greater variability, is shown for AP (*ii*) in main root bases (Fig. 4). As far as can be judged from the somewhat scattered points, the maximum concentration in the bases occurs at approximately 6 days after the maximum point in the root-tip curve. The most likely explanation of this behaviour is that AP (*ii*) production is going on in the main root tip and that the drift curve is a reflection on the one hand of auxin synthesis running concurrently with protein synthesis during active meristem growth and on the other hand of losses by degradation and/or transport which become apparent only when growth and synthesis slow down. The drift curve in the base could be largely a reflection of these changes subsequent to trans-

port of auxin from the tips. The 6 days' delay in the appearance of the maximum concentration might be interpreted as an outcome of a very sluggish transport of auxin from root tips. This seems unlikely. It is more probably caused by the 'buffering' effect of the much larger volume of mature tissue in which the translocated auxin is accumulating. It is not inconceivable that the downward drift in AP (*ii*) in maturing tips is largely the result of this slow outward translocation, although in the mature bases destructive metabolism

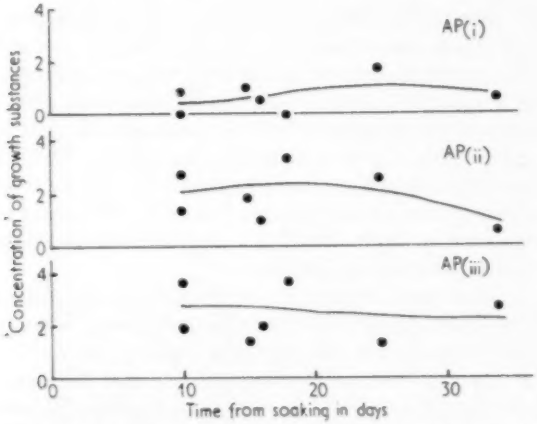

FIG. 5. Changes in the concentrations of the three principal acid auxins in whole lateral roots of *Vicia faba* in relation to the age of the parent root system.

must account for the greater part of the loss. It is also possible that lateral meristems which had not emerged at the time of sampling may also have contributed to the AP (*ii*) content of those bases and may to some extent account for the later maximum in the AP (*ii*) drift curve. These points need further direct study.

The behaviour of AP (*iii*) is quite different. In the first place it occurs in concentrations higher than those of AP (*ii*) in root tips but at lower concentrations in the bases. This accounts presumably for the concentrations of AP (*iii*) in whole-root systems being lower than those of AP (*ii*). In addition AP (*iii*) shows a tendency to increase in concentration as the root matures and stops growing and this is most marked in the root tip. There is some evidence here for the suggestion, thrown out in a previous paper (Audus and Lahiri, 1961) that AP (*ii*) and AP (*iii*) are interconvertible in the plant, since where net synthesis stopped with root growth, there seemed to follow an inverse correlation between the levels of the two compounds. Again it seems likely that the bulk of the AP (*iii*) is produced in the main tip meristem, from which it moves into the mature basal tissue.

AP (*i*) shows activities lower than AP (*ii*) or AP (*iii*) in both tips and bases, although the concentration is always higher in the tip. Trends are not so

clear-cut although there seems to be a progressive fall of concentration in both regions with ageing and the substance disappears completely when the root has stopped growing.

In lateral roots as a whole (Fig. 5) there is little indication, in these lower and somewhat scattered concentration values, of consistent drifts with ageing of the tissue.

(*c*) *The effects of decapitation of the main tap root*

The rather unexpected differences in the growth-substance content of lateral roots and main roots prompted a study of lateral tips and the effects on their auxin content of removal of the main tap-root meristem. It is well known that such a surgical operation may result, in actively growing roots, in an augmentation of the number of laterals produced per unit length of tap root and also in the greater growth of the existing laterals. This was borne out in our experiments, although it should be noted that the final weight of the complete root system was not significantly altered by this treatment. Since the growth of laterals can be thus influenced, it might be expected that the growth-substance balance could also be altered.

In this set of what must be regarded as preliminary experiments, a 5-mm. tip was removed from the main tap root at the 3-day stage, when the seedlings were transferred from sand to aerated water culture. Similar sets of control seedlings with intact tap-root meristems were grown at the same time. 9, 16, and 20 days later samples of roots were taken for analysis. All lateral root tips (terminal 5 mm.) were removed from both normal and treated roots and extracted separately. 5-mm. tips were also removed from the tap roots of control plants and discarded. The residual root bases minus the emerged meristems in both control and treated samples were then separately extracted.

The concentrations of the three main auxins in lateral tips and mature root tissue and the effects thereon on previous main root decapitation are shown in Fig. 6.

Taking first the situation in lateral-root tips, points have been plotted for the lateral tips of normal and treated root systems, and, for further comparison, the concentration drifts in normal tap-root tips of approximately the same age have been superposed. Since the main burst of lateral emergence takes place between 4 and 6 days, the lateral-root tips will therefore be approximately 5 days younger on the average than the tap-root tips of the same root system. In the graphs, therefore, there are drawn appropriate portions of the age drift curves of auxin from Fig. 3, shifted 5 days forward along the time axis.

With AP (*i*) there are no significant differences to be observed between lateral tips of control and decapitated roots and this auxin also seems to be at the same level as it is in the main tap-root tips.

AP (*ii*) in the lateral tips of normal roots is at a lower concentration than in the tap-root tip; indeed, in the older lateral tips (16 to 20 days) no AP (*ii*) could be detected. In the lateral tips of decapitated roots, however, the concentrations of AP (*ii*) are significantly higher. They approximate very closely

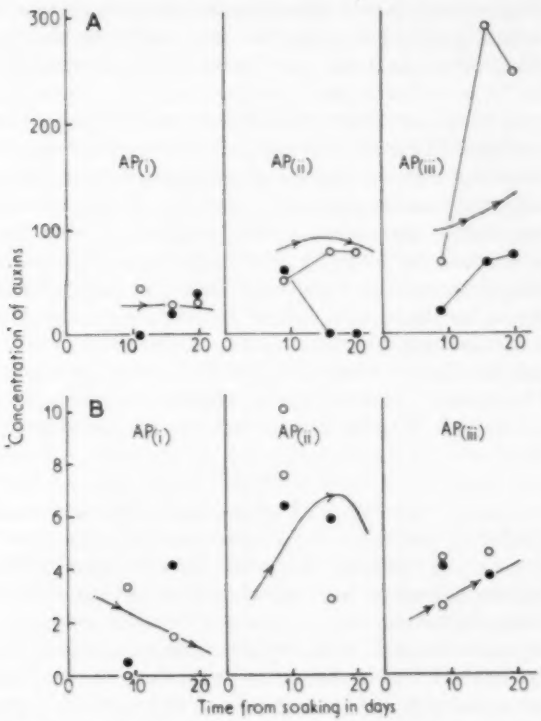

FIG. 6. The effects of main root decapitation on auxin behaviour in roots. Main root tip excised at 3 days.

A. Changes in 5-mm. lateral root tips.
—●—control root system.
—○—decapitated root system.
→——→—— shows the auxin drifts in main tap-root tips of approximately comparable age (from Fig. 3).

B. Changes in main tap-root base (minus main tip).
—●—control root system.
—○—decapitated root system.
→——→—— shows the auxin drifts in normal bases (from Fig. 4).

to, and show the same kind of drift as, the AP (*ii*) content of normal tap-root tips.

With AP (*iii*) the changes are even more striking. In the lateral tips of normal roots concentrations are about half those in normal tap-root tips but show the same upward trend with ageing. In decapitated root systems, however, the AP (*iii*) of the lateral tip is markedly increased, and in the most mature tips reaches concentrations about double those in normal tap-root tips. It is of interest that these changes in AP (*ii*) and AP (*iii*), which run very largely parallel, are not immediate on decapitation but are slowly established

as the laterals grow and mature. This suggests that changes in auxin metabolism of the lateral tips may be very indirectly related to the removal of the tap-root tip and might be partly a reflection of the increased growth of the laterals.

The effect of main root decapitation on the auxin content of root bases also confirms these ideas. Thus there are insignificant changes in AP (*i*) or AP (*iii*). In AP (*ii*), however, there is a significant increase in the concentration above that of normal root bases at 9 days, followed by a significant decrease at 16 days. The most likely explanation of this behaviour is that the increase at 9 days is the result of the greater number of new meristems induced in, but not yet emerged from, the decapitated root. As suggested earlier, such meristems would be the sites of AP (*ii*) production. Later, when all these meristems have emerged, the site of synthesis now being in the lateral tips, the content of AP (*ii*) would fall to low levels. These suggestions have still to be checked by detailed observations on meristem initiation and emergence, so that the chronology of these several events can be precisely correlated.

DISCUSSION

From these results the following points might be made concerning the possible role of these acid auxins in the growth-physiology of roots:

In the first place, the IAA-like auxin AP (*ii*) seems to be synthesized predominantly in the meristems, both apical and lateral. The amounts accumulating there seem to be very closely related to meristem activity, i.e. rate of production of new cells, as is shown by the close correlations with growth in the main tap-root tip, by the lower concentrations in the slower-growing normal lateral tips and by the increases there when growth is stimulated by main tap-root tip excision. It has already been shown (Lahiri and Audus, 1960) that AP (*ii*) is possibly an indole compound and the above correlations imply that it is produced from a precursor or precursors which are derived from the protein metabolic pool. In other words, it might prove to be a by-product of protein metabolism in these actively growing cells. To explain the loss of this compound from maturing meristems it is not necessary to evoke the development of a destructive enzyme system; in fact, the work of Pilet and Galston (1955), Pilet (1957) and Pilet and Siegenthaler (1959) has shown that IAA-oxidase, which is presumed mainly responsible for IAA destruction in plant tissues in the dark, has a very low activity in the root meristems of *Lens culinaris* as compared with its activity in mature tissue. The decline in the concentration of AP (*ii*) can be explained entirely in terms of its transport into mature tissue. Only in the oldest roots do the concentrations in the tip fall to equal those in the rest of the root. On the other hand there is undoubtedly a loss in the root as a whole (Fig. 2) and thus there must be a very active destruction in the mature tissues of the tap-root base. This too confirms the results of Pilet and his co-workers who found progressive increases in IAA-oxidase activity with maturation in *Lens* root cells. Whether AP (*ii*) could be degraded by this enzyme still remains to be seen.

The situation with AP (*iii*) seems different. Firstly, it exists at much higher activity levels than AP (*ii*) in main root meristems (Fig. 3) and also its concentrations do not show the same closely correlated drifts with meristem activity (Figs. 1 and 3). It also increases in concentration in all root tissues as the root system ages but this is most marked in the main tip meristem (Fig. 3), and also in lateral meristems when the main root tip has been removed (Fig. 6). The much higher concentration in the meristem would, on the face of it, suggest that it too, like AP (*ii*), is synthesized there and subsequently translocated to the mature tissue. But it seems not to be degraded to any extent in mature root tissue (Fig. 4) as is AP (*ii*). (The low value for 25-day-old roots in the preliminary experiments is exceptional and calls for further study.) The maintenance of a very steeply falling gradient of concentration from tip to base therefore suggests that translocation of AP (*iii*) is extremely slow as compared with that of AP (*ii*). In fact, most of this substance in mature cells could be accounted for on the assumption that it is merely the residue of the auxin produced during the meristematic phase of the cell's growth, and retained unchanged in the cell during extension and maturation. Only the very slight (and perhaps insignificant) rise of concentration in mature tissues would be due to translocation from the tip.

The third important difference between AP (*iii*) and AP (*ii*) is that its production does not seem to stop when growth of the meristem stops, since it accumulates in the tip when growth decreases. During active meristem growth, as has been suggested above, it has been continuously carried away from the meristem as the cells elongate and differentiate. This auxin has thus some of the properties of that hypothetical staling substance which Street (1954) has suggested might account for the cessation of cell division in excised roots grown in sterile culture. The fact that, unlike AP (*ii*), it is not destroyed may mean that its accumulation in the tip may bring it to a threshold concentration where it inhibits cell division. It is very interesting that Pilet (1951) observed a similar accumulation of *total* solvent-extractable auxin (assayed by the *Avena* curvature test) in the apical regions of roots of *Lens culinaris* as they grew from 8 to 22 mm. The concentration of auxin increased about 1,000 times (as calculated on IAA equivalence) in this period. But the situation may not be strictly comparable with that in *Vicia* roots; *Lens* does not seem to possess an auxin of the nature of AP (*iii*), since only 'accelerator α' (AP (*i*)?) and an IAA-like auxin (AP (*ii*)?) could be demonstrated (Pilet, 1958). Presumably the IAA-like auxin of *Lens* could be behaving there like AP (*iii*) of *Vicia* and might be causing the 'ageing' of the meristem. It would be most instructive to know what happens to the various growth substances in *Lens* root tips when the growth of the meristem slows down and finally stops.

The role of AP (*i*) is much more obscure. It too is probably produced in the meristem, as indicated by the much higher concentration there. It may move out with the developing cell or may be translocated as is AP (*ii*) and, like that substance, may be metabolized away in mature tissue. Since it does not appear to be an indole compound (Lahiri and Audus, 1960) it is unlikely that the

rough correlations between its concentrations in the meristem and that of AP (*ii*) are based on any direct metabolic connexions between the two substances.

The situation in lateral roots calls for special comment. As far as the rather scanty data go it seems reasonably clear that similar gradients of auxin concentration exist as in main tap roots. With AP (*i*) absolute concentrations are about the same as in the main root, but in normal lateral roots AP (*ii*) and AP (*iii*) are present in much lower amounts. The fact that, on main root decapitation, lateral growth increases and there is a correlated increase in the content of AP (*ii*) and AP (*iii*) suggests very strongly that the correlative repression of lateral growth by the main root apex is not mediated by these auxins. Another mechanism must be involved; release from inhibition allows both growth and auxin synthesis to proceed, although, in view of the suggested growth-independence of AP (*iii*)-synthesis in main root meristems, its rapid accumulation in lateral root tips is surprising. The situation somewhat resembles that in lateral buds whose development is correlatively depressed by the apical bud. As early as 1938 Ferman showed that such inhibited lateral buds of *Lupinus* contained much less auxin than similar buds which were growing. A similar phenomenon was shown by van Overbeek (1938) in *Pisum* seedlings. The similarity between these phenomena in lateral buds and lateral roots may be more than coincidental.

The final comment on these results concerns the possible growth activities of the main auxins at the concentrations existing in growing root cells. AP (*ii*) concentrations, if expressed in IAA equivalents, can rise to about 100 μg./Kg. in the root tip and to 5 μg./Kg. in the mature tissue. Since the root tips extracted contain cells already elongating, it seems highly probable that these elongating cells contain an AP (*ii*) concentration approaching the higher value, say about 50 μg./Kg. If AP (*ii*) had the same root-inhibiting activity as IAA, then this concentration in the extending cell (i.e. about 0·05 p.p.m.) would undoubtedly be supraoptimal, since IAA applied externally can inhibit growth of these root cells at concentrations as low as 0·001 p.p.m. But we have already seen (Lahiri and Audus, 1960) that AP (*ii*) is probably much less effective as a root inhibitor than IAA, and calculations suggest that the above concentration of AP (*ii*) would be equivalent, in terms of root-growth inhibition, to only 0·05 μg./Kg. of IAA. This would definitely be suboptimal for IAA, which, externally applied at this concentration, would stimulate root growth. Unfortunately it has not yet been shown that AP (*ii*) applied externally can stimulate root growth at this dilution, although it must be admitted that the root segments used for such tests may already contain optimal amounts of this substance.

The same argument might hold for AP (*iii*), which has a root-activity/shoot-activity ratio roughly similar to that of AP (*ii*). If, therefore, these two substances have any role to perform in controlling the normal extension growth of roots, then they may do so, as do the auxins of shoots, at a suboptimal level. Only when their production is very greatly increased, as in geotropic stimula-

tion (Audus and Lahiri, 1961), may they act as inhibitors, i.e. at the supra-optimal level.

AP (*i*) stimulated root extension in all concentrations so far tested (Lahiri and Audus, 1960). Its changes in relation to normal growth and experimental manipulation of the root are small and not so clear-cut as those of AP (*ii*) and AP (*iii*); further comment on its possible role would therefore be premature at this juncture.

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The Uptake of Growth Substances

III. INFLUENCE OF INDOLEACETIC ACID AND OTHER AUXINS ON THE UPTAKE OF 2,4-DICHLOROPHENOXYACETIC ACID BY *CHLORELLA*

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WITH FIVE FIGURES IN THE TEXT

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SUMMARY

When 2,4-dichlorophenoxyacetic acid, labelled with ^{14}C, is accumulated by *Chlorella pyrenoidosa*, an appreciable fraction, which increases with time, is held within the cell in a form which is not removed when the cells are placed in a solution containing the unlabelled compound. The accumulation of this fraction is dependent upon the supply of metabolic energy since it is affected by temperature, light, and the addition of inhibitors or citrate to the external medium.

The uptake of this metabolically accumulated 2,4-D follows a pattern which indicates that a single enzymic reaction is a predominant component of the uptake process. In the presence of the other auxins, including indoleacetic acid, 2,4,5-trichlorophenoxyacetic acid and 4-chlorophenoxyacetic acid, the uptake of 2,4-D is depressed and the changes suggest that there is competition at the site of this reaction. The nature of the competition is seemingly of the type where each compound serves as a substrate for the enzyme.

It may be significant that the extent to which the individual auxins compete with 2,4-D is in the same order as their general effectiveness as growth regulators for other species.

INTRODUCTION

WITH the object of elucidating the processes by which auxins exert control over growth and metabolism the pattern of uptake has interested a number of workers (for the earlier papers see review by van Overbeek, 1956). Since at least a portion of the auxin in the cell must be available for the critical reactions which control the observed responses to external applications of growth regulators, a knowledge of the uptake process and the factors which affect it should aid in an eventual understanding of the mechanisms of auxin action.

It has been noted for *Lemna minor* (Blackman, 1956; and Blackman, Sen, Birch, and Powell, 1959), for *Avena* (Johnson and Bonner, 1956) and for *Chlorella* (Wedding and Erickson, 1957) that the pattern of uptake for 2,4-dichlorophenoxyacetic acid (2,4-D) consists of at least two phases. In the case of *Chlorella* the initial phase is governed by a purely physical diffusion and

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an adsorption equilibrium with a half time of attainment of the order of 5 minutes. This process is freely reversible and, while a normal intact cell is essential, the process is not metabolic in nature. The second, and much slower, phase has been shown to exhibit at least some of the characteristics of a metabolic system and probably involves either the conversion of 2,4-D to some other compound which does not readily diffuse out of the cell, or a metabolic accumulation of 2,4-D behind a diffusion barrier within the cell. It may also be that both processes are involved either simultaneously or sequentially.

The present paper is concerned with this second, slower phase of the uptake of 2,4-D by *Chlorella pyrenoidosa* Chick. The approach has been to use 2,4-D, labelled in the carboxyl group with ^{14}C, and to apply the methods of enzyme kinetics in an analysis of the uptake process in intact cells. The second phase has been isolated by removing the readily diffusible or exchangeable 2,4-D prior to the assay of the amount taken up by the cell.

MATERIALS AND METHODS

Emerson's strain of *C. pyrenoidosa* Chick was cultured in nutrient solution as described by Wedding and Erickson (1957), save that the culture vessel was of one-litre capacity and the air supply was enriched with CO_2 to an approximate concentration of 3 per cent.

The cells were harvested 5 days after inoculation and a concentrated cell suspension prepared by centrifugal sedimentation. The entire culture was centrifuged, the packed cells washed by resuspending in 250 ml. of fresh nutrient solution, centrifuged again, and finally suspended in nutrient solution to a volume one-quarter of the original. This concentrated cell suspension was distributed in 2-ml. aliquots to conical flasks of 50 ml. capacity. In addition, each flask received 2 ml. of 0·04 M. K phthalate buffer adjusted to pH 5·5; 2 ml. of water, auxin or inhibitor solution made up to give the desired final concentration; and 2 ml. of labelled 2,4-D solution.

The flasks containing the *Chlorella* suspensions were placed in a water bath at 25° C. and were shaken at a rate of 80 cycles per minute. Illumination was provided by a bank of six 30-watt fluorescent tubes, fixed 12 inches above the surface of the bath.

At the end of the period selected for uptake the suspension was transferred to 15-ml. centrifuge tubes, the cells spun down, rinsed with 2 ml. of distilled water, and resuspended in 8 ml. of 0·01 M. K phthalate buffer (pH 5·5) containing unlabelled 2,4-D at a concentration ten times greater than that employed during the period of uptake. This suspension was returned to the original 50-ml. flasks and shaken on the water bath for an additional 30 minutes. On completion of this period the cells were again spun down, rinsed with 2 ml. of water, and finally transferred to aluminium planchettes with the aid of 1 ml. of propylamine, dried on the planchettes under infra-red lamps and the total activity of ^{14}C determined. From the original concentrated cell suspension 20-ml. aliquots were filtered, and the cells dried and weighed for the calculation of 2,4-D content on a dry weight basis.

EXPERIMENTAL RESULTS

Factors affecting the course of uptake. For periods of uptake as short as 10 minutes, Chlorella reversibly adsorbs 2,4-D to an extent which at pH 5·5 may amount to as much as 10 per cent. of the total in the cells (Wedding and Erickson, 1957). The nature of this binding is non-metabolic, but shows a marked response to changes in external pH, suggesting that proteins or similar compounds provide the binding sites. In order to eliminate this adsorbed fraction, as well as any freely diffusible 2,4-D, from the cells before the

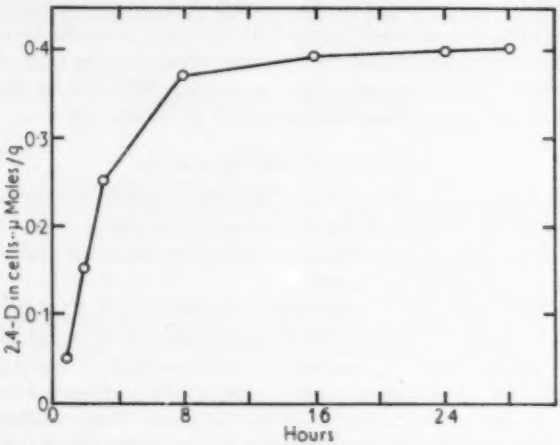

FIG. 1. The accumulation with time of non-diffusible, non-exchangeable 2,4-D ('metabolic' fraction) by *Chlorella* cells, given continuous light at 25° C.

remainder was assayed, the conditions leading to the maximal removal of this fraction were studied. It was found that over the concentration range used, cells transferred to a solution of unlabelled 2,4-D ten times more concentrated than the original labelled solution reached equilibrium in 20 minutes. No detectably greater loss could be demonstrated by increasing the volume of the external solution above 4 ml.

Accordingly a standard 'exchange' procedure, which also includes removal by diffusion, was adopted: an initial washing in water, followed by 30 minutes in 8 ml. of unlabelled 2,4-D in buffer (approximately forty times the cell volume) and a second washing in water.

This procedure was first employed to follow the accumulation of the non-diffusible, non-exchangeable form of 2,4-D by *Chlorella* over a period of 28 hours. It may be seen from Fig. 1 that the capacity of *Chlorella* to take up 2,4-D from a 10^{-4} M. solution is saturated only slowly, the half time is approximately 3 hours as compared with about 5 minutes for the initial phase of non-metabolic diffusion (Wedding and Erickson, 1957). It is probable that the upward slope of the curve in Fig. 1 from 8 to 28 hours is at least in part

due to the formation of new cells rather than a continued accumulation in existing cells. Over the period of this experiment the non-diffusible non-exchangeable fraction of the total 2,4-D in the cells changed from 18 per cent. at 1 hour to 49 per cent. at 28 hours.

The slow rate and irreversible nature of the uptake process indicated that it was not due to simple diffusion and a series of experiments was undertaken to demonstrate the relation of this process to a supply of metabolic energy.

Effects of inhibitors. The effects of several metabolic inhibitors on the amounts of 2,4-D accumulated by *Chlorella* in 2 hours from a 5×10^{-4} M. solution are summarized in Table I. These data are from different experiments

TABLE I

The effects of several inhibitors on the amount of 2,4-D remaining in the cells of Chlorella *after they had been allowed to take up labelled 2,4-D (5×10^{-4} M.) for 2 hours and then transferred to a solution containing unlabelled 2,4-D and incubated for an additional 30 minutes*

Inhibitor (Conc. 5×10^{-4} M.)	2,4-D in cells μMoles/g. dry wt.		
	Control	Treated	Per cent. of control
Iodoacetate . . .	0·543	0·137	25
Dinitrophenol . . .	0·534	0·269	50
Fluoride	0·486	0·440	90
Arsenate	0·536	0·491	92

in which the level of uptake of untreated cells varied somewhat, but when the amount taken up in the presence of each inhibitor is expressed as a percentage of that found in the comparable controls, the compounds are seen to vary widely in their effects. At equal concentrations iodoacetate is more effective than the others in reducing uptake, causing a 75 per cent. reduction. The presence of 2,4-dinitrophenol at 5×10^{-4} M., which is an uncoupling concentration for *Chlorella* (Wedding and Black, 1961), halves the uptake of 2,4-D. Fluoride and arsenate in similar concentrations have relatively little effect, but under these conditions neither of these inhibitors affects the respiration of *Chlorella* appreciably; an indication that if they do penetrate into the cell it is at a very slow rate.

Effects of light and temperature and citrate. Other evidence that a metabolic component is involved in the uptake of 2,4-D is forthcoming from the changes induced by light, temperature and the presence of respirable substrate. Table II shows that when 2,4-D is taken up in darkness the amount which remains in the cells is reduced by more than 40 per cent. If the cells are pre-treated by placing them in the dark for 24 hours in an aerated nutrient solution, and then allowed to take up 2,4-D in the dark, the amount in the cells is less than 50 per cent. of the quantity which accumulates in the light. These facts suggest that the primary effect of light is through provision of substrates for an energy-consuming process.

Temperature also had a pronounced effect on the uptake of 2,4-D in darkness: a change from 25° C. to 12° C. causes almost a 50 per cent. reduction. This reduction corresponds to a Q_{10} of 1·67 for the uptake process over this range of temperature. The addition of 10^{-2} M. citrate to the medium leads to an increase of 46 per cent. in the uptake in the light, *vide* Table II. It may be added that this concentration of citrate causes an approximate threefold increase in oxygen uptake by *Chlorella*.

TABLE II

The effects of light, temperature, and citrate on the amount of 2,4-D remaining in the cells of Chlorella *after they had been allowed to take up labelled 2,4-D (5×10^{-4} M.) for 2 hours and then transferred to solution containing unlabelled 2,4-D and incubated for an additional 30 minutes*

Treatment	2,4-D in cells	
	μMoles/g. dry wt.	Per cent.
Light—1,000 foot candles	0·482	100
Dark during uptake and exchange	0·321	67
Dark during uptake and exchange preceded by 24 hours in darkness	0·223	47
Uptake at 25° C.	0·609	100
Uptake at 12° C.	0·320	53
Uptake from buffer alone	0·401	100
Uptake from buffer plus 0·01 M. citrate	0·586	146

The effects of inhibitors, temperature, light, and added substrate all support the postulate that a large component, if not the whole of the mechanism controlling the net uptake, is metabolic in nature and that the accumulation of 2,4-D comes about as a result of some process driven by respiratory energy.

The effects of other auxins on the uptake of 2,4-D. Alterations in the response to one auxin due to the presence of a second have been observed many times, e.g. McRae and Bonner, 1952; Hoffman, 1953; Henderson and Deese, 1954; Foster, McRae and Bonner, 1955; Gowing, 1956; Lockhart and Weintraub, 1957. Data bearing on the influence of one auxin on the uptake of another are more scanty but Reinhold in this laboratory (1954) showed that the uptake of indoleacetic acid either by segments of pea epicotyl or carrot disks was inhibited by 2,4-D. Indoleacetic acid (IAA) was also found by Johnson and Bonner (1956) to inhibit the uptake of 2,4-D by *Avena* coleoptile sections, but the inhibition of uptake was smaller than the effect on the rate of extension growth. More recently Blackman (1961) has reported that the addition of phenoxyacetic acid (POA) to the external solution causes a reduction in the uptake of 2,4-D by *Avena* coleoptiles but that the decrease is less for stem segments of *Gossypium*. It was therefore decided that it would be of interest to examine the influence of a range of concentrations of IAA and other auxins on the uptake of 2,4-D by *Chlorella*.

When the fraction of 2,4-D in the cell which is in diffusion and adsorption

equilibrium with the external solution is removed by the 'exchange' procedure, the relationship between the residual labelled carbon and the external concentration of 2,4-D is a hyperbolic function, as is shown in the lowest (no IAA) line of Fig. 2. This double reciprocal or Lineweaver–Burk plot of uptake as a function of concentration gives an excellent fit with $r = 0{\cdot}92$ and makes it possible to apply the methods of enzyme kinetics (Reiner, 1959) to the study of the effects of other auxins on the uptake of 2,4-D.

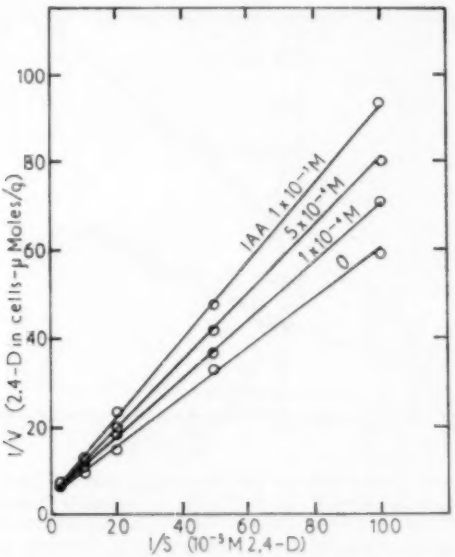

FIG. 2. Inter-relationships between the metabolic uptake of 2,4-D by *Chlorella* over 2 hours and the external concentrations of 2,4-D and IAA. The reciprocals of the amounts of 2,4-D in the cells (1/V) have been plotted against the reciprocals of the external concentrations of 2,4-D (1/S). The lowest line represents conditions where no IAA was present in the external solution, for the remaining lines the concentrations of IAA are indicated. The cells were given continuous light at 25° C.

The common intercept of the 1/V *vs.* 1/S lines for the uptake of 2,4-D in the presence of increasing amounts of IAA shows that the inhibitory effect of IAA is overcome by high concentrations of 2,4-D, with the result that there is a change in the slope, but not in the intercept, of the lines. This relationship indicates that the effect of IAA on the uptake of 2,4-D is of a type which is generally called 'competitive'.

Another method of evaluating competition consists in the plotting of 1/V against I (inhibitor, or in this case, IAA concentrations) (Friedenwald and Maengwyn-Davies, 1954). The family of straight lines produced in this way (Fig. 3), intersect at a point to the left of the ordinate but above the abscissa, again demonstrating competitive inhibition. The line for 1×10^{-4} M. 2,4-D

has essentially zero slope, suggesting that the condition of infinite substrate is being approached.

Although these experiments seemingly demonstrate that IAA reduces the metabolic uptake of 2,4-D by competing for a limiting reaction in the uptake process, they reveal little about the mechanism of this competition. Reiner (1959) suggests methods for the resolution of several types of inhibition which on the basis of the relationships shown here for 2,4-D and IAA could be

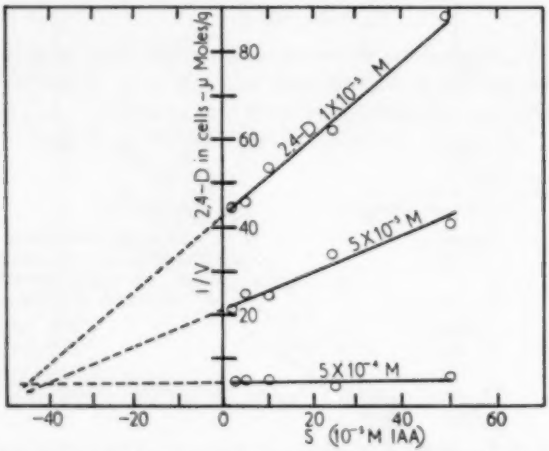

FIG. 3. Inter-relationships between the metabolic uptake of 2,4-D by *Chlorella* and the external concentration of IAA (1/S). The reciprocals of the amounts of 2,4-D in the cells (1/V) have been plotted against the external concentrations of IAA. For each line the external concentration of 2,4-D is indicated. The cells were given continuous light at 25° C.

classed as 'competitive'. Considering the known similarities in the action of the two compounds and their steric relationships, the type discussed by Reiner which would appear to be the most probable is the one he classifies as 'partial inhibition, type b'; that is, both the inhibitor and the substrate are capable of mutually replacing each other on the enzyme. The primary criteria for identification of this type of inhibition are that the double reciprocal plot must be linear, the rate of the reaction when saturated with substrate must be the same in the presence or absence of inhibitor, and complete saturation with inhibitor must completely inhibit the reaction. The data available for the effects of IAA on the uptake of 2,4-D satisfy the first two of these requirements, but conclusive evidence for complete inhibition is not forthcoming.

At least two explanations for this inability to inhibit completely the uptake of 2,4-D by IAA seem possible. One is that the solubility limits of IAA do not permit the required inhibitor concentration to be attained. The other is that only part of the 2,4-D in the cells has been taken up through the

IAA-inhibited reaction and that the remainder is present in the cells as a result of some other process, which is not affected by IAA.

Another means of identifying the type of competition is given by Reiner which consists of plotting I (inhibitor concentration) against the ratio $i/1-i$ (fraction of activity inhibited/fraction of residual or uninhibited activity). The results of treating the present data in this way are shown in Fig. 4. From a theoretical consideration of the case of partial inhibition in which both

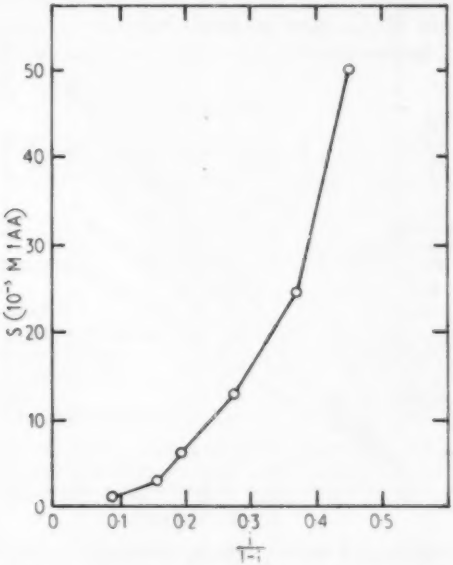

FIG. 4. The inter-relationship between the external concentration of IAA (S) and the ratio of the metabolic fraction of 2,4-D accumulated to the residual fraction. For further explanation see text.

inhibitor and substrate can replace one another on the enzyme, Reiner shows that the relationship of I *vs.* $i/1-i$ should be curvilinear, with a negative intercept if the final portion of the curve is extrapolated back to the I axis. The relationship in Fig. 4 is of this type. If one accepts a mechanism for the inhibition by IAA of the uptake of 2,4-D in which either can replace the other on the active site, the most probable explanation is that both are substrates for the reaction and that either can be converted to the final product, in this case absorbed auxin which is non-diffusible and non-exchangeable. A simple method for the analysis of this system is not available, and although it could be approached by a study of mutual inhibition using labelled forms of both compounds, this has not so far been done.

If both IAA and 2,4-D are metabolically accumulated by *Chlorella* through a common mechanism, then other auxins, particularly those substituted

phenoxyacetic acid compounds whose level of activity is similar to that of 2,4-D should also utilize the same mechanism and accordingly should compete with 2,4-D.

The effect of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) on the uptake of 2,4-D by *Chlorella* is illustrated by the double reciprocal plot given in Fig. 5. Here again the second auxin has reduced the amount of 2,4-D taken up in a

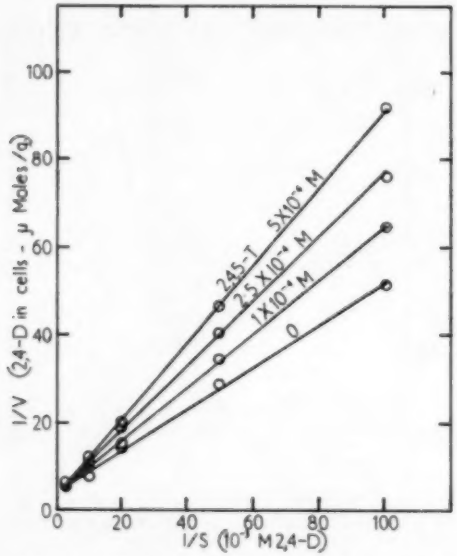

FIG. 5. Inter-relationships between the metabolic uptake of 2,4-D by *Chlorella* cells over 2 hours and the external concentrations of 2,4-D and 2,4,5-T. The reciprocals of the amounts of 2,4-D in the cells (1/V) have been plotted against the external concentrations of 2,4-D (1/S). The lowest line represents the condition where no 2,4,5-T was present in the external solution, for the remaining lines the concentrations of 2,4,5-T are indicated. The cells were given continuous light at 25° C.

way which points to a competition of the two compounds for a common reaction site in the pathway of metabolic accumulation. The degree of inhibition caused by 5×10^{-4} M. 2,4,5-T, as indicated by the value of 1/V corresponding to 100 (10^{-5} M. 2,4-D) on the 1/S scale, is greater than that brought about by the same concentration of IAA. In fact the effects are only approximately equal when the concentration of IAA is raised to 1×10^{-3} M.

Other analogues of 2,4-D have been tested in the same way, including 4-chlorophenoxyacetic acid (4-CPA), 2-chlorophenoxyacetic acid (2-CPA), phenoxyacetic acid (POA), and 2,6-dichlorophenoxyacetic acid (2,6-D). Of this series it was clearly established that 4-CPA competes with 2,4-D in the same way as IAA and 2,4,5-T, although it seems to be less effective. POA and 2-CPA, while they have only slight inhibitory effects on the uptake of

2,4-D, appear to be competitive, i.e. the small amounts of inhibition recorded can be countered by an increase in the concentration of 2,4-D. On the other hand, 2,6-D induces no significant inhibition over a concentration range of 10^{-5} to 10^{-3} M. 2,4-D and 10^{-4} to 10^{-3} M. 2,6-D.

For a number of reasons it is difficult to carry out an accurate comparison of the relative inhibitory powers of a number of auxins which reduce the metabolic uptake of 2,4-D. Firstly, there is some variation in the uptake of 2,4-D by *Chlorella* from one experiment to another. Secondly, a replicated experiment with several concentrations of both 2,4-D and the inhibitory auxin is so large that only one auxin can be evaluated at a time. Thirdly,

TABLE III

Effects of other auxins, each at a concentration of 5×10^{-4} *M., on the metabolic uptake of 2,4-D by* Chlorella *from a* 5×10^{-5} *M. solution during 2 hours in the light at 25° C.*

Treatment	2,4-D in cells	
	μMoles/g. dry wt.	Per cent.
None (Control)	0·0725 Aa*	—
Phenoxyacetic acid	0·0715 Aa	99
2,6-dichlorophenoxyacetic acid	0·0713 Aa	98
2-chlorophenoxyacetic acid	0·0688 Aa	95
4-chlorophenoxyacetic acid	0·0485 Bb	67
Indoleacetic acid	0·0470 Bb	65
2,4,5-trichlorophenoxyacetic acid	0·0379 Bc	52

* Figures followed by the same letter are not significantly different. Small letters denote significant difference at the 5 per cent. level: capitals denote a significant difference at the 1 per cent. level.

kinetic theory (Reiner, 1959) casts grave doubt on the validity of the use of K or Km as an 'affinity constant' or measure of the closeness of fit of the substrate to the enzyme. In consequence comparisons of inhibitor effectiveness on the basis of the relative values of Km may be suspect.

In view of these considerations a direct comparison of the inhibitory power of the selected auxins has been made by determining the degree to which the uptake of 2,4-D is reduced by a single concentration of the other auxins. This type of experiment can be carried out with five replicates on a single lot of cells and should provide a more valid measure of relative effectiveness.

In one experiment a comparison was made of the effects of IAA, 2,4,5-T, 4-CPA, 2-CPA, POA and 2,6-D, all at a concentration of 5×10^{-4} M. on the uptake of 2,4-D and the amounts taken up in the presence and absence of each auxin are listed in Table III. The data have been analysed by the 'multiple range' test (Snedecor, 1956). It is to be observed that POA, 2,6-D and 2-CPA have not depressed the rate of uptake; 2,4,5-T is the most effective inhibitor while IAA and 4-CPA occupy an intermediate position. In our experiments a tenfold greater concentration of 2,4,5-T is required to bring about a 50 per cent. reduction in the uptake of 2,4-D. In this connexion it has also been

observed by Johnson and Bonner (1956) that the overall inhibitory effect of other auxins on the uptake of 2,4-D by *Avena* coleoptiles is not great.

DISCUSSION

These experiments have shown that in *Chlorella* the fraction of 2,4-D which cannot be readily removed from the cell by diffusion or desorption amounts to about one-quarter of the total taken up after 2 hours and about half at the end of 28 hours. This high level of retention is in contrast with the findings for *L. minor* with both 2,4-D (Blackman *et al.*, 1959) and triiodobenzoic acid (Blackman and Sargent, 1959) where much of the 2,4-D originally absorbed moves out into the culture solution after transference. Again, with both intact plants or stem segments of *Gossypium* (Blackman, 1961) or potato tuber disks (Bennet-Clark and Wheeler, 1959) significant amounts of the 2,4-D accumulated by *Gossypium* or the IAA taken up by the potato disks are subsequently lost, since with time the net rate of uptake changes from a positive to a negative value. This reversal has not been found with *Chlorella* in periods of up to 36 hours, when the external hydrogen ion concentration is maintained constant.

The 2,4-D retained by *Chlorella* may accumulate unchanged behind a diffusion barrier, or the compound may be metabolized to some other, less readily diffusible form. This question is under investigation and will be the subject of a later paper. Whatever the mechanism of the accumulation, its response to metabolic inhibitors, to temperature, light, and citric acid indicates that it is dependent upon a supply of metabolic energy. The rectangular hyperbolic relationship of this metabolic uptake to the external concentration of 2,4-D is indicative that the uptake process, although it may consist of many sequential reactions, is probably limited primarily by one of the linked reaction steps in the process. Other interpretations are possible, indeed the failure of dinitrophenol or IAA wholely to inhibit uptake can be cited as evidence for the operation of more than one system.

It has been demonstrated that the effects of the presence of other active auxins such as 2,4,5-T, IAA and 4-CPA upon the uptake of 2,4-D can be interpreted as competitive and the nature of the induced changes lends strong support to the view that all are suitable as substrates for the uptake reaction and are capable of mutually displacing one another at the active site of the limiting reaction. This postulate does not carry the implication that they must all eventually arrive at the same point or all be effective in controlling a growth process. The limiting step may be one which occurs early in the chain of events which eventually leads to a growth response and each compound may be shunted to its own individual route at some later step. In this connexion it is significant that those auxins which compete successfully with 2,4-D can be classed as having high physiological activity in regulating growth. The failure of 2,6-D, 2-CPA, and POA at an equivalent concentration to depress the uptake of 2,4-D may therefore be associated with their much lower level of physiological activity.

ACKNOWLEDGEMENTS

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Auxin Antagonistic Effects of Auxin Precursors of the Substituted Amide Type

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WITH ONE PLATE CONTAINING THREE FIGURES, AND THREE FIGURES IN THE TEXT

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SUMMARY

Cucumber seeds were germinated in Petri-dishes for 6 days with different doses of a number of N-monosubstituted amide derivatives of some chlorophenoxy-alkane-carboxylic acids. Doses of the amides studied were roughly equivalent to 0·1, 1, 10, 100, and 1,000 p.p.m. in water. The gross morphological effects of the amide treatments were studied, both with and without the addition of 1 and 10 p.p.m., respectively, of the corresponding carboxylic acid. The amides studied were the N-methyl-, N-ethyl-, N-propyl-, N-iso-propyl-, N-butyl-, and N-benzyl-amides of 2,4-dichlorophenoxyacetic acid (2,4-D), α-(2,4-dichlorophenoxy)-propionic acid (2,4-DP), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and α-(2,4,5-trichlorophenoxy)-propionic acid (2,4,5-TP).

Thirteen of the twenty-one amide derivatives studied antagonized the auxin-type plant-growth-regulating effects of their parent carboxylic acid. The antagonisms observed involved not only a decrease in the stimulatory effects of the externally applied auxin acids (counteractions of the abnormal swellings of different plant parts), but also reduced significantly their inhibitory effects, i.e. the inhibitions of growth in length of the hypocotyl and of the roots. Since some auxin-type stimulatory effects were observed with all of the amides studied, it is suggested that the thirteen amide derivatives play a double role as plant growth regulators in cucumber tissues: (1) to a certain extent they are transformed by plant enzymes to the corresponding carboxylic acid, i.e. to auxin; and (2) the amides themselves exert anti-auxin effects.

INTRODUCTION

THE first paper devoted to structure-activity relationships in auxin-type chemicals (Koepfli *et al.*, 1938) has already suggested that all those compounds which do not possess a free carboxyl group are inactive *per se*, but that their auxin activity is entirely due to acidic transformation products. This suggestion has since been supported by indirect evidence presented by a number of workers; at the same time some difficulties have been encountered, for example, the different concentration-activity curves for the assumed precursor and its parent auxin (cf. Michel 1957), and the greater maximum responses in some tests with the precursor than with its postulated transformation product (e.g. Linser 1956).

Most of the latter observations have been tentatively explained as an additional growth effect of the precursor itself. For this reason we have

developed a new test procedure (Südi *et al.*, 1960) for studying the plant-growth regulating activity of a number of potential precursors of chlorophenoxy-alkane-carboxylic acids (Südi *et al.*, 1959; Josepovits *et al.*, 1960). This 'seedling morphology test' can be used to determine the concentration of auxins of the latter type. The essential feature of our test is that it takes into consideration all the morphological reactions of treated seedlings. We wish to stress again that, although not all the individual components of the overall growth effect of auxin-type chemicals are auxin-specific yet the quantitative correlations between them are certainly highly characteristic of a given auxin. It was assumed, therefore, that the overall morphological effects of an auxin precursor would not resemble those of the corresponding auxin, if, in addition to the auxin effect expected of its transformation product, the precursor evoked its own additional responses through another mechanism.

It has indeed been observed that the overall morphological effect of a great number of the precursor-type derivatives of chlorophenoxy-alkane-carboxylic acids which have been studied are not the same as those produced by the corresponding carboxylic acid, at least with some of the species studied (Südi *et al.*, 1959; Josepovits *et al.*, 1960). Certain similarities have also been noted in the same test between the overall morphological effects of a number of assumed auxin precursors on the one hand, and some anti-auxins (or auxins with a strong anti-auxin component in their physiological activity) on the other hand (Südi, unpublished). These similarities suggested a double action for these precursors in treated plants, namely: (1) auxin effect of the enzymatically produced parent auxin, and (2) anti-auxin effects exerted by the untransformed molecules of the precursor. Our first results along these lines are described in the present paper, and these give strong support to the above hypothesis.

MATERIALS AND METHODS

The compounds used were highly purified preparations of the N-methyl-, N-ethyl, N-propyl, N-isopropyl-, N-butyl-, and N-benzyl-amides of 2,4-dichlorophenoxyacetic acid (2,4-D), α-(2,4-dichlorophenoxy)-propionic acid (2,4-DP), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and α-(2,4,5-trichlorophenoxy)-proprionic acid (2,4,5-TP); of the N-propylamides only the 2,4-D-derivative was available. The compounds were prepared in our laboratory; their physical constants, and the purification processes used, &c., have already been described (Josepovits *et al.*, 1960).

Cucumber (*Cucumis sativus* var. 'Erfurti felhosszu') seedlings were used as the plant material. All the experiments reported were based on the seedling morphology test (Südi *et al.*, 1960) referred to in the introduction. Cucumber seeds (20 seeds per dish) were germinated for 6 days in 12 cm. Petri-dishes containing 12 ml. aqueous solution at $24{\cdot}5 \pm 0{\cdot}5^{\circ}$ C. in the dark. The limited water-solubilities of the amides in question made difficult their solution directly in water. Solutions in acetone were therefore prepared, and filter-paper disks supporting the germinating seeds were impregnated with them.

The following amide treatments were applied (for details see Josepovits *et al.*, 1960):

dose 1 (impregnation with 1 per cent. acetonic solution): roughly equivalent to the amount of active material contained by 12 ml. aqueous solution of 1,000 p.p.m. concentration;

dose 2 (impregnation with 0·1 per cent. acetonic solution): roughly equivalent to 100 p.p.m.;

dose 3 (impregnation with 0·01 per cent. acetonic solution): roughly equivalent to 10 p.p.m.;

dose 4 (impregnation with 0·001 per cent. acetonic solution): roughly equivalent to 1 p.p.m.; and

dose 5 (impregnation with 0·0001 per cent. acetonic solution): roughly equivalent to 0·1 p.p.m.

Three series of six Petri-dishes were prepared, each representing all the above five doses of the amides together with an untreated blank. In one of these series distilled water was used to moisten the filter-paper, while the other two were moistened with aqueous solutions containing 1 and 10 p.p.m. of 2,4-D, respectively. Thus an experimental unit, devoted to the study of the effects of one of the substances, consisted of 3×6 dishes, each representing one of the possible combinations with 2,4-D.

Evaluation of the interactions in most experiments was done by visual observations only. Observations included all the perceptible morphological reactions of the seedlings, but interactions were first sought in the effects on lengths of the hypocotyl, the main root, and the secondary roots; and also in the effects on root and hypocotyl swellings. Detailed records of the observations were supplemented by photographs of the treated seedlings. Experiments were repeated at least three times; the morphological observations were found to be very reproducible.

Laborious and time-consuming measurements were undertaken only with N-butyl-(2,4-dichlorophenoxy)-acetamide. Seedling weight was measured with a torsion balance to the nearest mg., after the removal of the seed coat and cotyledons. Root and shoot lengths were measured to the nearest mm. Results summarized in Figs. 4 to 6 are average values, based on three separate experiments in good agreement, every single value being derived from the measurement of 50 to 60 seedlings. Standard errors were computed for each mean value. However, $\pm 2s_{\bar{x}}$ for the butylamide-free treatments only are shown on the graphs, since the coefficient of variation was fairly constant. In some dubious cases further analyses of significance were made with the '*t*-test'. The results obtained are to be found in the text.

RESULTS

(*a*) *Visual observations.* The effects on the growth of cucumber seedlings of the amides studied alone and in combination with 1 and 10 p.p.m. of the corresponding carboxylic acid cannot be described in full detail. The usefulness of the applied procedure will be illustrated by photographs of responses

to one of the 21 compounds studied (Figs. 1 to 3). A summary of all the most important visual observations is given in Table I.

In Figs. 1 to 3 representative seedlings from the samples of 20 show the effects of treatments in which 2,4-D-butylamide was combined with 2,4-D.

TABLE I

Antagonisms by different doses of 21 amide derivatives of growth inhibitions caused by the corresponding chlorophenoxy-alkane-carboxylic acid, of hypocotyl length (h), main root length (m), and lengths of the secondary roots (s), respectively. (Symbols in brackets denote inconspicuous counteraction of the inhibition)

		Dose of the amide				
		(5)	(4)	(3)	(2)	(1)
2,4-dichlorophenoxy-						
N-methyl acetamide	+ 1 p.p.m. 2,4-D	—	(m), (s)	(m), (s)	m, s	—
	+ 10 p.p.m. 2,4-D	—	—	—	m, s	—
N-ethyl acetamide	+ 1 p.p.m. 2,4-D	—	(m), (s)	(h), m, s	m, s	—
	+ 10 p.p.m. 2,4-D	—	—	(h), (m), s	—	—
N-propyl acetamide	+ 1 p.p.m. 2,4-D	—	—	(m), (s)	m, s	—
	+ 10 p.p.m. 2,4-D	—	—	(m), (s)	h, m, s	—
N-isopropyl acetamide	+ 1 p.p.m. 2,4-D	—	—	—	m, s	m, s
	+ 10 p.p.m. 2,4-D	—	—	—	m, s	m, s
N-butyl acetamide	+ 1 p.p.m. 2,4-D	—	—	(h), m, s	m, s	m, s
	+ 10 p.p.m. 2,4-D	—	—	h, m, s	h, m, s	h, m, s
N-benzyl acetamide	+ 1 p.p.m. 2,4-D	—	—	m, s	m, s	m, s
	+ 10 p.p.m. 2,4-D	—	—	s	s	s
2,4-dichlorophenoxy-						
N-methyl propionamide	+ 1 p.p.m. 2,4-DP	—	—	—	m, (s)	—
	+ 10 p.p.m. 2,4-DP	—	—	—	(m), s	—
N-ethyl propionamide	+ 1 p.p.m. 2,4-DP	—	—	—	m, (s)	—
	+ 10 p.p.m. 2,4-DP	—	—	—	(m), s	—
N-isopropyl propionamide	+ 1 p.p.m. 2,4-DP	—	—	—	—	—
	+ 10 p.p.m. 2,4-DP	—	—	—	—	—
N-butyl propionamide	+ 1 p.p.m. 2,4-DP	—	—	—	—	—
	+ 10 p.p.m. 2,4-DP	—	—	—	—	—
N-benzyl propionamide	+ 1 p.p.m. 2,4-DP	—	—	—	—	—
	+ 10 p.p.m. 2,4-DP	—	—	—	—	—
2,4,5-trichlorophenoxy-						
N-methyl acetamide	+ 1 p.p.m. 2,4,5-T	—	—	h, (m), (s)	m	—
	+ 10 p.p.m. 2,4,5-T	—	—	—	—	—
N-ethyl acetamide	+ 1 p.p.m. 2,4,5-T	—	—	m, s	—	—
	+ 10 p.p.m. 2,4,5-T	—	—	(m), (s)	(m)	(m)
N-isopropyl acetamide	+ 1 p.p.m. 2,4,5-T	—	—	—	—	—
	+ 10 p.p.m. 2,4,5-T	—	—	—	—	—
N-butyl acetamide	+ 1 p.p.m. 2,4,5-T	—	—	—	(m)	(m)
	+ 10 p.p.m. 2,4,5-T	—	—	—	—	—
N-benzyl acetamide	+ 1 p.p.m. 2,4,5-T	—	—	(h), m, s	m, (s)	—
	+ 10 p.p.m. 2,4,5-T	—	—	h, m, s	h, m, s	—
2,4,5-trichlorophenoxy-						
N-methyl propionamide	+ 1 p.p.m. 2,4,5-TP	—	—	—	—	—
	+ 10 p.p.m. 2,4,5-TP	—	—	—	—	—
N-ethyl propionamide	+ 1 p.p.m. 2,4,5-TP	—	—	m, s	m, s	(s)
	+ 10 p.p.m. 2,4,5-TP	—	—	—	m	—
N-isopropyl propionamide	+ 1 p.p.m. 2,4,5-TP	—	—	—	—	—
	+ 10 p.p.m. 2,4,5-TP	—	—	—	—	—
N-butyl propionamide	+ 1 p.p.m. 2,4,5-TP	—	—	—	—	—
	+ 10 p.p.m. 2,4,5-TP	—	—	—	—	—
N-benzyl propionamide	+ 1 p.p.m. 2,4,5-TP	—	—	—	—	—
	+ 10 p.p.m. 2,4,5-TP	—	—	—	—	—

Fig. 1 shows the effects of the butylamide without added 2,4-D, in doses (3), (2), and (1). Dose (3) is practically ineffective (equal to the untreated control). Effects are first apparent only in dose (2), viz., reduced lengths of main root, secondary roots, and hypocotyl. Most of the root tips (i.e. those which were in contact with the filter-paper) are swollen. In dose (1) a further

significant reduction in hypocotyl length can be observed, while root lengths are not further reduced. Stimulatory effects evoked by dose (1) consist not only of root-tip swellings, but also of hypocotyl swellings which are apparently associated with the appearance of more or less differentiated adventitious roots.

The effects of doses (4) to (1) of 2,4-D-butylamide combined with 1 p.p.m. of added 2,4-D are seen in Fig. 2. The ineffective dose (4) did not bring about any perceptible alteration in the reaction of cucumber seedlings to 1 p.p.m. 2,4-D; the first two seedlings in Fig. 2 (dose (4)) serve therefore as controls, with which the effects of increasing doses of the butylamide can be compared. It is seen that even dose (3), although completely devoid of any activity by itself, antagonizes some of the inhibiting effect of 1 p.p.m. 2,4-D to a remarkable extent (see lengths of the main root and of the secondary roots). The antagonism in root length is even stronger with doses (2) and (1), but the butylamide apparently could not counteract the inhibition in hypocotyl length caused by 2,4-D since its own inhibiting effect was even stronger (cf. Fig. 1). If we compare the effects of doses (2) and (1) in Fig. 2 with the corresponding doses without 1 p.p.m. of 2,4-D (Fig. 1) from any point of view, it is seen that no difference is caused by the addition of 1 p.p.m. of 2,4-D to these higher butylamide doses.

Fig. 3 illustrates the effects of increasing butylamide doses in the presence of 10 p.p.m. 2,4-D. Dose (4) is equivalent to a control as in Fig. 2, because modifications of the effects of 10 p.p.m. 2,4-D were not discernible at this concentration of the amide. Inhibitions of growth in length of hypocotyl, main root, and secondary root were all relieved to about the same extent by doses (3) to (1) of the amide except main root length in dose (3). In the latter combinations the overall morphological effect of the butylamide component increasingly predominates, though the 2,4-D is not entirely ineffective, e.g. the types of root branching and root swelling are more like the effects of 2,4-D than of the amide.

The main points of the above observations might be summed up as follows:

(i) The inhibitory effects of 1 and 10 p.p.m. 2,4-D are significantly counteracted by doses (3) to (1) of its butylamide derivative, although dose (3) is completely inactive by itself.

(ii) Antagonisms could be observed only in those growth responses in which the amide component was less active than the acid.

In general the above statements hold for other acid/amide combinations studied, where antagonistic effects could be observed. Some features of the latter observations are summarized in Table I, where only inhibitions of growth in length caused by 1 and 10 p.p.m. of the corresponding chlorophenoxy-alkane-carboxylic acids are considered, since they present the most critical aspects of possible antagonisms. Specific antagonisms of 2,4-D are indicated in all the combinations of 2,4-D amides, but only some of the 2,4,5-T-amides (i.e. the methyl-, ethyl-, and benzyl- derivatives) were active against 2,4,5-T. The results suggest that the methyl side chain of the α-propionic acid analogues affects unfavourably the antagonistic activity of the amides

in question, since only the methyl- and ethyl-amides of 2,4-DP, and the ethylamide of 2,4,5-TP, have shown antagonisms against 2,4-DP, and 2,4,5-TP, respectively.

(*b*) *Some quantitative characteristics of the 2,4-D-butylamide/2,4-D inter-*

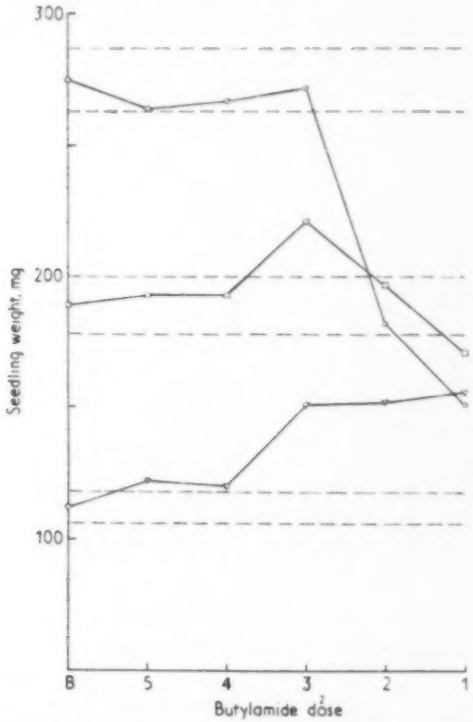

FIG. 4. Interaction between different doses of N-butyl-(2,4-dichlorophenoxy)-acetamide and 1 and 10 p.p.m. of 2,4-D on the weight of cucumber seedlings. (B: butylamide-free blank.)

Broken lines $\pm 2s_{\bar{x}}$ for the butylamide-free treatments;
○ without added 2,4-D;
□ plus 1 p.p.m. 2,4-D;
△ plus 10 p.p.m. 2,4-D.

actions. The above results, obtained by the visual evaluation of the effects on seedling morphology, have been checked by exact measurements. The 2,4-D-butylamide/2,4-D combinations discussed above were chosen for this purpose. Treatments and incubation were the same as above but measurement of the growth effects was different: seedling weight, hypocotyl length, and main root length were measured. The latter aspects were chosen because (1) they made exact measurements possible, and (2) treatments with 1 and 10 p.p.m. of 2,4-D inhibited these aspects of growth (see Figs. 4 to 6).

Seedling weights are shown in Fig. 4. The control value of 275 mg. per seedling is seen to be reduced to 239 and to 112 mg. by 1 and 10 p.p.m. of 2,4-D respectively. Increasing doses of the butylamide without added 2,4-D (open circles, Fig. 4) reduce seedling weight significantly only in doses (2)

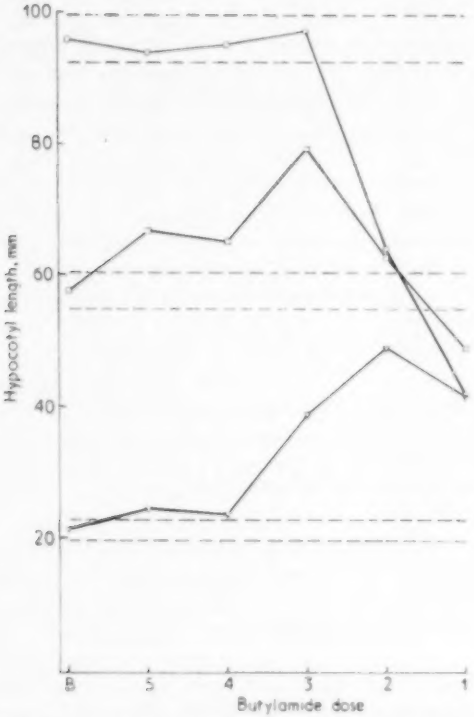

FIG. 5. Interaction between different doses of N-butyl-(2,4-dichlorophenoxy)-acetamide and 1 and 10 p.p.m. of 2,4-D on the length of the hypocotyl of cucumber seedlings. (B: butylamide-free blank.)

Broken lines $\pm 2s_{\bar{x}}$ for the butylamide-free treatments;
○ without added 2,4-D;
□ plus 1 p.p.m. 2,4-D;
△ plus 10 p.p.m. 2,4-D.

and (1). When the same butylamide doses were combined with 1 p.p.m. 2,4-D (open squares, Fig. 4), the otherwise ineffective dose (3) relieved significantly the inhibition caused by 1 p.p.m. 2,4-D, while in doses (2) and (1) the inhibitory effects were equal to or greater than those caused by 2,4-D alone. The third curve (open triangles, Fig. 4) represents combinations involving 10 p.p.m. 2,4-D. In amide doses (5) and (4) seedling weight values are somewhat higher than with 10 p.p.m. 2,4-D alone. A *t*-test showed the difference to be significant at the 5 per cent. level ($t = 2{\cdot}181$ for 84 degrees

of freedom) with dose (5), and not significant ($t = 1{\cdot}955$, d.f. $= 106$) for dose (4). Since both values are at just about the 5 per cent. level of significance the difference between the effects of dose (5) and dose (4) cannot be regarded as significant. However, most significant antagonisms of the inhibitions caused

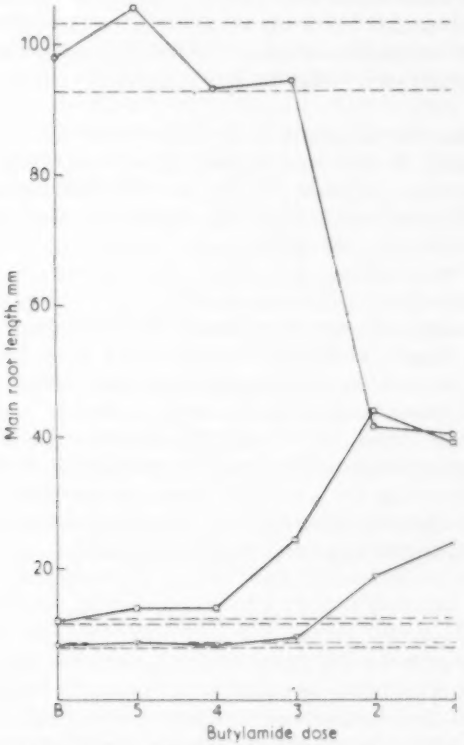

FIG. 6. Interaction between different doses of N-butyl-(2,4-dichlorophenoxy)-acetamide and 1 and 10 p.p.m. of 2,4-D on the length of the main root of cucumber seedlings. (B: butylamide-free blank.)

Broken lines $\pm 2s_{\bar{y}}$ for the butylamide-free treatments;
○ without added 2,4-D;
□ plus 1 p.p.m. 2,4-D;
△ plus 10 p.p.m. 2,4-D.

by 10 p.p.m. 2,4-D are shown by doses (3) to (1) of butylamide. On the other hand, absolute growth values apparently cannot surpass those found with the amide alone.

Seedling weight in these experiments is evidently determined mainly by the weight of the hypocotyl. Since hypocotyl weight is determined by diameter as well as length, it is evident that data on hypocotyl length (Fig. 5) reflect changes in a more simple growth process; in consequence these changes are

more pronounced. In addition, the only noticeable difference between Figs. 4 and 5 is that, in respect of hypocotyl length, significant antagonisms against the inhibitory effect of 1 p.p.m. 2,4-D are shown also by butylamide doses (5) and (4) (open squares, Fig. 5). The latter interactions could not be detected by visual observation (see Table I). The effects of doses (5) and (4) on the inhibition caused by 10 p.p.m. 2,4-D are of a doubtful significance, just as in the case of seedling weights. The following values were obtained by the *t*-test: $t = 2{\cdot}182$ (d.f. = 84) for dose (5), and $t = 1{\cdot}632$ (d.f. = 106) for dose (4).

Even more clear-cut antagonisms are shown in the effects on length of the main root (Fig. 6). In this respect doses (2) and (1) of the amide are less inhibitory than even 1 p.p.m. 2,4-D, and therefore antagonisms were clearly established with these combinations. On a per-cent. basis, the antagonisms here (doses (2) and (1) of the amide with 1 p.p.m. 2,4-D) were the greatest encountered in these studies; they represented 250 per cent. stimulations as compared to growth in 2,4-D alone. But here again the inhibitory effects of the combined treatments were never weaker than with the butylamide alone.

Two further features of the data of Figs. 4 to 6 must also be mentioned. They are, first, the visually unrecognized stimulation of root growth by dose (5) of the amide (open circles, Fig. 6), which proved to be significant at the 5 per cent. level ($t = 2{\cdot}01$; d.f. = 106); and, secondly, the puzzling fact that in dose (1) seedling weight (Fig. 4), and hypocotyl length (Fig. 5) are both significantly higher with 1 p.p.m. 2,4-D (open squares) than without it (open circles). With 111 degrees of freedom, $t = 2{\cdot}656$, and $t = 2{\cdot}306$ were obtained for seedling weight, and hypocotyl length, respectively.

DISCUSSION

When we compare the exact data of Figs. 4 to 6 with the results of visual observations reported in the first part of the paper (cf. Table I and Figs. 1 to 3), it is seen that measurements reflect more sensitively the situation. However, the main points of the conclusions which can be drawn are the same, and perhaps even more instructive in the case of visual observations, where every single component of the overall morphological growth effect is simultaneously considered.

The results presented show that, with cucumber seedlings, 13 of the 21 amide derivatives studied have shown effects antagonistic to the action of the corresponding chlorophenoxy-alkane-carboxylic acid. Since the antagonisms observed involved remarkable reductions of the inhibitory effects of the corresponding carboxylic acid, it is suggested that the amides in question exert highly specific, competitive antagonisms, i.e. they should be regarded as true anti-auxins. This suggestion is supported by the more or less close structural analogy between 2,4-dichloroanisol, the classical example of those anti-auxins without a carboxyl ring (cf. Leopold 1955) and the amides studied (e.g. the methylamide of 2,4-D).

On the other hand, when discussing the diverse types of growth effects on cucumber seedlings of the most strongly antagonistic compound (i.e. 2,4-D-butylamide, see Figs. 1 to 3) it was pointed out that seemingly auxin-specific growth stimulations were always present as components of the overall growth effects of the amides in question. If we accept the well-supported hypothesis (see Veldstra, 1953) that a carboxyl group or perhaps an acidic group in general, is essential for auxin activity of a molecule, then the latter auxin-specific effects can be explained only by the partial conversion by plant enzymes of the anti-auxin amide to the auxin carboxylic acid.

Based on the results presented above it is even possible to give a rough estimate of the extent to which the latter conversion might take place. It was shown, in connexion with Figs. 1 to 3 (cf. also Figs. 4 to 6) that (*a*) the addition of 1 p.p.m. of 2,4-D to dose (2) of the butylamide did not evoke any noticeable new effect, while 10 p.p.m. 2,4-D altered remarkably the appearance of the seedlings, and (*b*) even 10 p.p.m. 2,4-D added to dose (1) of the butylamide only slightly altered the reaction of cucumber seedlings to the amide. On the other hand, it has already been stated in connexion with the sensitivity of the applied test procedure (Südi *et al.*, 1960), that no clear differences in the effects can be seen by doubling the concentration of the active auxin. Therefore, the highest concentration of added 2,4-D which seems to be ineffective when combined with the given dose of the amide might be regarded as an indication of the amount of acid produced from amide in treated plants.

If it is assumed that anti-auxins of this type are characterized by their partial conversion to auxin, it is easily seen that the competitive nature of their antagonistic effects cannot be convincingly demonstrated by the kinetic analysis introduced by McRae and Bonner (1952). However, the data presented here seem to demonstrate the competitive nature of the observed antagonisms, i.e. the anti-auxin character of 13 of the amides studied.

We wish to stress that by the experiments described we attempted to discover whether there are any auxin analogues which are auxin precursors as well as auxin antagonists. Some characteristics of the experimental conditions chosen are unsuitable from some points of view; for example, the extended period of treatment before observations were made, the high concentrations required and the complex nature of the growth system in intact seedlings. We have not tried to explain, therefore, some of the complications, as indicated e.g. by Figs. 4 and 5, where 1 p.p.m. 2,4-D is shown to relieve the inhibition caused by the highest dose of N-butyl-(2,4-dichlorophenoxy) acetamide.

However, a short reference should be made to some further observations (Südi, unpublished); it was then found that the auxin-antagonist activity of the amides studied varies with plant species (Südi, unpublished). 2,4-D-butylamide, for instance, antagonized the effects of 2,4-D only on cucumber; the same interaction could not be demonstrated with any of the 20 other species studied, which were those investigated by Josepovits *et al.* (1960)

together with a *Cucurbita* ('squash'). This observation would certainly eliminate some objections that might be raised to our interpretation of the results. On the other hand, differences between the sensitivity of different species to auxin precursors might be augmented by the selective auxin-antagonist activity of these compounds (cf. Table I, and Table 5 of Josepovits *et al.*, 1960).

To the best of our knowledge the present paper is the first indication that compounds capable of being degraded to auxin by plant enzymes might exert direct anti-auxin effects. While it is obvious that most anti-auxins cannot be converted to auxins by plant enzymes, some of them might certainly be transformed—e.g. γ-phenylbutyric acid, the auxin-antagonistic activity of which was described by Skoog *et al.* as early as 1942. On the other hand, it is clear that since the structural requirements of anti-auxin activity are not precisely known, all the compounds classified in the literature as auxin precursors may, in theory, have anti-auxin properties.

It remains for further studies to establish the extent to which this possibility is in fact realized, using other plant and compound combinations. In conclusion we should like to point out one of the most interesting problems raised by the observations described. The physiological role of precursor-type natural indole derivatives may be merely as precursors of the active indoleacetic acid, as indeed has been assumed so far; but they might also have a significant role to play by virtue of their possible anti-auxin activity.

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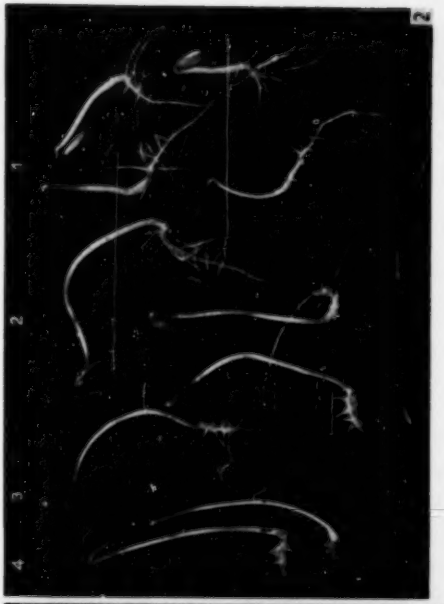

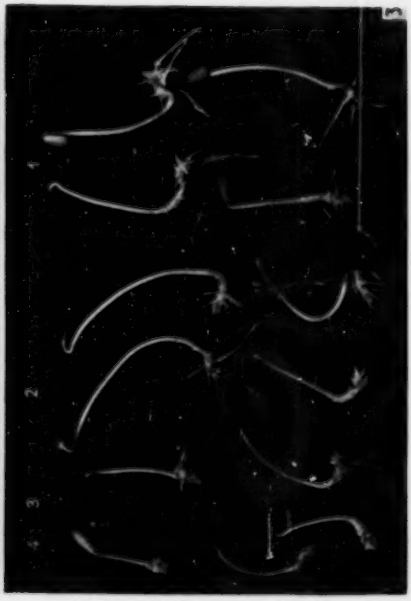

FIG. 1. Effects of different doses of N-butyl-(2,4-dichlorophenoxy)-acetamide on cucumber seedlings. Doses (3), (2), and (1).

FIG. 2. Effects of 1 p.p.m. 2,4-D on cucumber seedlings in the presence of different doses of N-butyl-(2,4-dichlorophenoxy)-acetamide. Doses (4) to (1).

FIG. 3. Effect of 10 p.p.m. 2,4-D on cucumber seedlings in the presence of different doses of N-butyl-(2,4-dichlorophenoxy)-acetamide. Doses (4) to (1).

J. SÚDI, GY. JOSEPOVITS, and GY. MATOLCSY—PLATE I

The Behaviour of Phloridzin in the Coleoptile Straight-growth Test

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WITH THREE FIGURES IN THE TEXT

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SUMMARY

Phloridzin was identified as one of the two inhibitors of coleoptile-section extension in the acid fraction of extracts of apple shoots. The tips of the sections showed a characteristic flaccidity and staining at concentrations of 200 p.p.m. and above. Sections which had been inhibited by phloridzin did not recover so well after washing and transfer to sodium β-indolylacetate as did those which had been in contact with an inhibitor from plum shoots.

Phloridzin was more effective as an inhibitor than its breakdown products phloretin, phloroglucinol, *p*-hydroxyhydrocinnamic acid, or *p*-hydroxybenzoic acid.

The scanty knowledge of the role of phloridzin in plant metabolism is briefly discussed.

INTRODUCTION

In the course of a survey of paper chromatograms of ether extracts of stems and leaves with the coleoptile straight-growth test, marked differences were observed between the inhibitors of cell extension present in plum and apple shoots. In the plum a single acid inhibitor was found, of which some of the biological characteristics have been described elsewhere (Barlow *et al.*, 1961*a*). An important feature of this substance is that coleoptile-section extension can be strongly inhibited for several hours and yet the sections appear quite healthy; when washed with water they are capable of considerable extension in auxin solutions. In the apple-shoot extracts at least two acid inhibitors often occur, one with an Rf in butanol-propanol-ammonia-water (5:5:1:1) of about 0·75, the other of about 0·25. The former, like the plum inhibitor, has no visible ill effects on the coleoptile section, but the latter causes a characteristic flaccidity at the ends of the section (and indeed at any other wound), and regrowth of sections after washing is not nearly so marked. This inhibitor is usually the only one found in the acid fraction of apple-root extracts.

Recently, the examination of the Rf of this inhibitory substance in a number of solvent mixtures, together with various chromogenic spray reactions, has shown it to be the glycoside phloridzin, and this paper describes the behaviour of the pure substance in the coleoptile straight-growth test.

MATERIALS AND METHODS

Chromatography. The solvent mixture used in this laboratory in routine chromatographic procedures (butanol-propanol-water-ammonia 5:5:1:1) is not a good one for phloridzin, and several of the more satisfactory standard mixtures were used on various occasions in identifying the inhibitor as phloridzin, and in demonstrating its breakdown products. The recognized chromogenic sprays were employed.

Bioassay was by the wheat-coleoptile straight-growth test used in this laboratory for some years, test solutions being held in rotated tubes of small volume, usually closed (Hancock and Barlow, 1953; Barlow *et al.*, 1957). In certain experiments the tubes were left open, which can safely be done without the solution running out during horizontal rotation.

'*End flaccidity*'. The condition referred to by this phrase is quite distinct, but difficult to describe or illustrate: a zone of flaccid cells extends from the cut ends of the section to a brownish stained line which sharply divides the flaccid from the healthy tissue. The damage can be definitely recognized when the zone is about 0·25 mm. long, and at its maximum it seldom exceeds 2·5 mm. The basal end of the section is usually affected first.

In most experiments the flaccidity of the sections was rated visually on a 0–10 scale, 10 being complete flaccidity, and 4 the characteristic end-flaccidity induced by phloridzin; in a few trials the lengths of the flaccid zone and of the whole section were measured with an ocular micrometer at a magnification of ×7·5.

RESULTS

Inhibition and end flaccidity. The effects of a range of phloridzin concentrations from 1 to 1,600 p.p.m. (a nearly saturated solution at room temperature) have been examined in a number of section tests, of which Table I is an example.

TABLE I

Percentage inhibition of coleoptile sections by phloridzin solutions and the percentage of section length flaccid*

	Phloridzin p.p.m.									
	0	100	200	400	600	800	1,000	1,200	1,400	1,600
Inhibition	0	14	24	41	47	55	64	66	71	73
Flaccidity	0	0	4	12	18	23	30	35	41	42

* $\left(100 \times \frac{\text{increase in length in water} - \text{increase in solution}}{\text{increase in water}}\right)$.

At concentrations below 100 p.p.m. coleoptile response is variable, growth sometimes being slightly promoted; flaccidity of the section-ends, however, appears very consistently at 200 p.p.m. and increases with concentration.

Section extension in sodium β-indolylacetate (NaIAA) is also reduced by phloridzin (Fig. 1). In this test, at each level of auxin, all phloridzin

concentrations reduced growth; conversely, at all concentrations of phloridzin, NaIAA increased extension, even though at 800 p.p.m. phloridzin the auxin-induced growth was slight. These results did not show unequivocally whether added NaIAA offset the end-flaccidity due to phloridzin, but other experiments indicate a slight reduction of such symptoms by the growth promoter.

Certainly in extracts containing natural phloridzin and promoter the two substances sometimes interfere on the chromatogram; if, for example, an extract of apple roots is chromatographed in butanol-propanol-ammonia-water (5:5:1:1), assay with coleoptile sections reveals different patterns according to the loading (Fig. 2). At 0·5 g. dry wt. of roots per cm. width of paper there is good separation of phloridzin, Rf *c.* 0·25, and promoter, Rf *c.* 0·45; at 1 g./cm. there is a slight displacement towards higher Rf values, but still good separation, but at 2 g./cm. further spread of the phloridzin zone has masked the promoter, which, however, slightly ameliorated the flaccidity of the ends of the sections.

Inhibition and regrowth of sections. The contrast between the behaviour of sections upon transfer from plum inhibitor or phloridzin to auxin has been commented on in the introduction, and is illustrated in Fig. 3. The lower two lines show the inhibition brought about by phloridzin and plum inhibitor (concentration of the latter being in arbitrary units). The upper two lines show the regrowth of these sections after 16 hours in inhibitor, thorough washing in running tap water,[1] and 20 hours in 5 p.p.m. NaIAA; the tubes were open all the time, so that there were no secondary effects of the kind described elsewhere (Barlow *et al.*, 1961*b*). The units compare the growth of sections after inhibition with that of sections after water:

$$100 \times \frac{Lt - Lw}{Lw}$$

where *Lt* is the increase in length after transfer of treated sections, and *Lw* the increase in length after transfer of sections originally in water.

It is evident that, for a given degree of inhibition greater than about 20 per cent., sections recover their ability to extend far better after plum inhibitor than after phloridzin. Failure after phloridzin is not due to an increase in the proportion of flaccid length in the second period offsetting the extension of undamaged cells, since measurement has shown that there is practically no additional flaccidity in the second period.

Breakdown products of phloridzin. Börner (1959) has shown that bacteria in soil are able to break down phloridzin to phloretin, which in turn yields phloroglucinol and *p*-hydroxyhydrocinnamic acid, the latter finally giving rise

[1] A fine glass jet protrudes through a circle of stiff nylon gauze so that on insertion into the tube containing sections to be washed the tip of the jet reaches nearly to the bottom, and the gauze closes the end. Tap water is led to the jet by a rubber tube with a by-pass so that the supply can easily be pinched off when inserting or removing the jet; the rate of supply is adjusted so that in 10 sec. washing *c.* 25 ml. of water flow through the tube over the sections. The tube is sucked dry with a water pump and the NaIAA solution added. This method is far quicker than that previously described (Hancock, 1959).

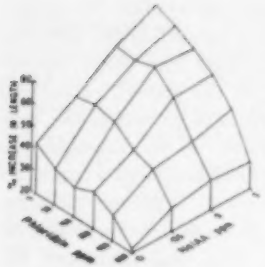

FIG. 1. Percentage increase in length of coleoptile sections in mixtures of phloridzin (increasing concentrations from back to front), and sodium β-indolylacetate (NaIAA) (increasing concentrations from front to back).

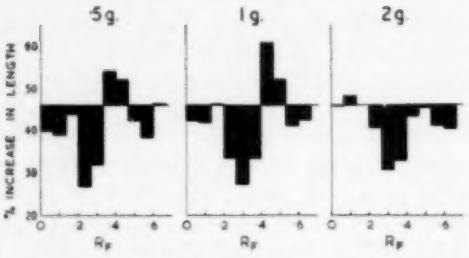

FIG. 2. Histograms showing the response of coleoptile sections to the first ten segments of chromatograms (divided into fifteen segments from starting line to the solvent front) loaded with extract from 0·5, 1·0, or 2·0 g. of dry apple root per cm. width of paper.

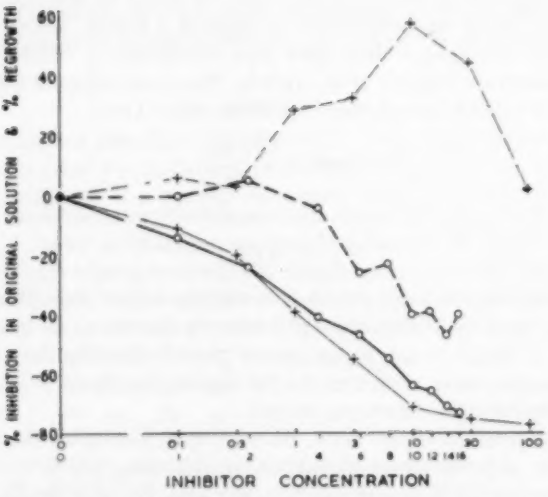

FIG. 3. Graphs showing inhibition of the growth of coleoptile sections (solid lines) due to phloridzin (— o —), at concentrations shown in the lower scale in hundreds of p.p.m., or to a natural plum inhibitor (— + —) on an arbitrary upper scale. Regrowth of the sections after washing and transfer to 5 p.p.m. NaIAA is shown with the same symbols and broken lines.

to *p*-hydroxybenzoic acid. In the present experiments it was also possible to show by chromatography that solutions of phloridzin which had been incubated with coleoptile sections or juice contained a number of these breakdown products. It was possible that one or more of these compounds may have been responsible for the phloridzin effect, rather than that material itself.

Each of the pure substances mentioned above was therefore examined at a similar range of concentrations to those used for phloridzin (except that phloretin was only soluble in water plus a trace of ethanol to the extent of 100 p.p.m.). None produced as much inhibition as phloridzin itself, and even at the highest concentrations no flaccidity of sections was observed except for traces in phloretin. It is of interest that when once-tested phloridzin solutions are retested in open tubes, thereby avoiding the secondary effects of microbial activity reported elsewhere (Barlow *et al.*, 1961*b*), they are in fact less inhibitory than when used fresh, presumably due to breakdown of phloridzin.

At the first step in decomposition the sugar moiety of the glycoside is split off, and it was thought possible that the slight growth promotion of low concentrations of phloridzin might be due to this extra sugar, as no sucrose is normally added to our test solutions; furthermore, Street and Lowe (1950) found that the inhibition by phloridzin of excised root growth could be offset by increasing the sugar concentration; in the present coleoptile test, however, a series of phloridzin concentrations with or without sucrose at 0·5 to 2 per cent. yielded response curves of similar shape, giving no suggestion of any interaction between the glycoside and the sugar.

Although no systematic survey was undertaken to compare the effect of phloridzin on coleoptile sections with that of related compounds or other inhibitors, a number have been tested (some much earlier than the present studies, in other connexions.) Table II summarizes the results.

It should be pointed out that the values given for the maximum percentage inhibition are only approximate, and in some cases based on only a single test or over a limited concentration range; but they give some indication of the great variation in the inhibition of coleoptile sections by different substances. In the first group phloridzin stands out as the most potent inhibitor, and the only one to cause marked end flaccidity; in the second group coumarin is an effective inhibitor above 10 p.p.m., but *o*-coumaric acid and umbelliferone inhibit only at very high concentrations. The respiration inhibitors and the other compounds in the third group were employed in connexion with 'regrowth' experiments, and it is of interest that the first three behaved like the plum inhibitor in causing inhibition which was readily relieved by washing and regrowing in NaIAA, while the next four had a much less completely reversible effect. Phloridzin, in this respect, was intermediate in action; few of the other compounds in the table have been tested in this manner.

In the neutral fraction of extracts of apple leaves and of young internodes an inhibitor occurs which produces typical end flaccidity; at least in the internode extract this would appear not to be phloridzin, since, although on heavily loaded chromatograms in our routine solvent mixture this material and

phloridzin have similar Rf's, at lighter loading (cf. Fig. 2), and particularly in certain other solvent systems, the two can be fairly widely separated.

TABLE II

Comparison of the effect of some glycosides, coumarins, and other inhibitory materials upon wheat coleoptile sections

Compound	Concentrations tested (p.p.m.)	Lowest concentration for definite inhibition	Maximum per cent. inhibition	Flaccidity
1. *Glycosides, aglycones and breakdown products*				
Phloridzin	0·01–1,600	50–100	75	'End'
Phloretin	0·01–100†	(50)§	5	V. slight 'end'
p-hydroxyhydrocinnamic acid	2·5–1,000	500	35	0
Phloroglucinol	0·01–1,000	(100)	12	0
p-hydroxybenzoic acid	0·01–1,000	(250)	12	0
Arbutin	2–1,000	—	0	0
Amygdalin	2–1,000	—	0	0
Naringenin•	2–250†	50	35	Slight general
Salicin	2–1,000	—	0	0
2. *Coumarin and related cinnamic acid derivatives*				
Coumarin	0·01–1,000	10–100	85	0
o-coumaric acid	0·01–1,000	250	85	'End' at 500, general and complete at 1,000
p-coumaric acid	0·01–100	100	10	0
Chlorogenic acid	0·01–1,000	1,000	? 5	0
Aesculin	0·01–1,000	1,000	? 5	0
Umbelliferone	0·01–1,000	1,000	50	0
3. *Respiration inhibitors and other miscellaneous compounds*				
Iodoacetic acid	50–200	100	30	0
2,4-DNP	1–25	5	30	0
Na azide	5–100	25	60	0
Na arsenite	10–200	10	60	—
KCN	6–6,500	30	95	—
Crystal Violet	0·001–100	10	60	0
$CuSO_4$	0·1–1,000	10	65	0
Ethanol	0·1–10,000	(250)	40	0
'Myrobalan inhibitor'	0·005–500‡	0·5	90	0

• A natural inhibitor from dormant peach buds described by Hendershott and Walker (1959).

† Limit of solubility in water with trace of ethanol.

‡ Solid only partially purified.

§ Values are bracketed where the response curve was not well defined; two values are given when the curves were definite, but varied from test to test.

DISCUSSION

These studies arose during a survey of natural growth substances in apple and plum shoots, in which differences were noted in the behaviour of coleoptile sections in the various inhibitors. The identification of one of the two

acid inhibitors in the apple as phloridzin widened the field of interest considerably, since the role of this substance in the metabolism of the apple is still very uncertain (e.g. Hutchinson *et al.*, 1959, is no more conclusive than Harvey, 1925, thirty-four years earlier).

That Luckwill (1957) was able to state definitely that his *Malus* inhibitor 1 was not phloridzin, although running in much the same position on some of his chromatograms (e.g. Figs. 3 and 4, Rf 0·2 to 0·3, though not in Fig. 1, Rf 0·45), was probably due to differences between his extraction procedure and ours. He used dry ether on dry plant material, with a return to an anhydrous final extract after separating the acidic fraction. Phloridzin is only very slightly soluble in ether, and we consider its presence in quantity in our extracts to be due to the use of wet ether on fresh material, with perhaps some emulsification by other constituents of the extract.

There seems to be little direct connexion between the amount or concentration of phloridzin in various apple tissues, and seasonal or other variations in growth (Priestley, 1960), yet the rapid incorporation of labelled carbon into phloridzin when supplied either as $^{14}CO_2$ or in certain elaborated substrates suggests that it is metabolically very active; once formed in the apple leaf, however, it appears to be rather inert, and certainly cannot be utilized by isolated leaf disks (Hutchinson *et al.*, 1959). Nevertheless, phloridzin can be readily broken down by enzymes of the polyphenoloxidase type in various plant tissues or extracts (Marrè, 1950*d*) (which did not include apple), by various apple tissues, possibly with changes during the season (Anon., 1960), and by micro-organisms in the soil (Börner, 1959).

In animal physiology phloridzin inhibits carbohydrate uptake by preventing phosphorylation of sugars (see Street and Lowe, 1950, for references), and this has led various workers to test phloridzin in plants for similar action. Street and Lowe (1950) overcame the phloridzin-induced inhibition of growth of excised roots by raising the sugar concentration of the medium; but we did not find such relief with sucrose on wheat coleoptile sections, and nor did Thimann and Marrè (1954) with oat sections, using various sugars and sugar-phosphates. These authors, however, conclude that phosphorylation is not a feature of sugar uptake by plant cells, and that it was not for this reason that phloridzin inhibited section extension. Marrè suggests that the inhibition of coleoptiles and roots of intact oat seedlings is due to a slowing down of cell division as well as elongation (1950*c*), and that ultimately the effect is mediated by the inhibition of dehydrogenase systems in the plant (both by phloridzin and phloretin) (1953).

Phloridzin, however, has promoting as well as inhibitory effects on plant metabolism: Marrè (1950*a*) showed that photosynthesis in *Elodea* was almost doubled under its influence, and oxygen consumption halved; starch synthesis was not affected (1950*b*), and it seems that the promoting effect is on the photochemical reaction itself (Marrè and Servattaz, 1955).

Whether phloridzin brings about these many effects only when introduced into plants to which it is foreign, or whether similar activities may occur in

the apple-tree cannot at present be stated, and in view of the considerable quantity of phloridzin there, it would seem important to settle the question. In certain investigations the characteristic reaction of the wheat coleoptile section may make it a useful test object.

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The Minimum Intercellular-Space Carbon-dioxide Concentration in Leaves of the Palm *Phoenix reclinata*

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WITH ONE FIGURE IN THE TEXT

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SUMMARY

Leaves of *Phoenix reclinata* respond both to high temperature and to water-strain with sudden and marked increase in their minimum intercellular-space carbon-dioxide concentration. The leaves of *Phoenix reclinata* which had been grown in the open country did not suffer a significant after-effect from being exposed to experimentally imposed water-strain.

INTRODUCTION

RECENT investigations by Heath and Orchard (1957), Rees (1958, 1961), Heath and Meidner (1957, 1958, 1961), and Meidner and Heath (1959) have dealt with midday closure of stomata as influenced by high temperature and by water-strain. These investigations showed that high temperature and water-strain can cause increases in the intercellular-space CO_2-concentration.

Leaves of *Coffee arabica* and of *Allium cepa* were found to respond to high temperature by a sudden and discontinuous increase in their minimum intercellular-space CO_2-concentration, but these two species did not respond in a similar manner to water-strain. By comparison leaves of wheat, variety 'Charter', and of maize (several varieties) responded to water-strain with increases in their minimum intercellular-space CO_2-concentration, but did not respond similarly to high temperature. From these results it appeared therefore that stomatal closure, in so far as it operates via an increase in the internal CO_2-concentration of the airspace system of the leaves, may be brought about either by high temperature or by water-strain. Rees's work on the oil palm showed that for this species both factors, water-strain and high temperature, might be operative. In his field experiments, however, both these factors were interrelated. An experiment with leaves of the palm, *Phoenix reclinata* (oil palm not being available in Natal) was therefore carried out and the two factors were tested separately.

APPARATUS AND METHODS

The apparatus used was very similar in construction to that described by Heath and Meidner (1961); only minor modifications were made to suit local circumstances.

1. *The influence of temperature on the minimum intercellular-space CO_2-concentration, Γ*

The method used for the measurement of the influence of temperature on Γ-values was similar to that employed by Heath and Orchard (loc. cit.). Four leaves were used; each leaf was used for four experimental runs. During each run four temperature treatments were applied in random order, so that a total of 64 readings was obtained. The temperatures used were 25° C., 30° C., 33° C., and 40° C., the illumination was 1,400 f.c., and the vapour-pressure deficit of the circulating air-stream was 12 mm. Hg.

2. *The influence of water-strain on the minimum intercellular-space CO_2-concentration, Γ*

For the influence of water-strain on Γ-values the method employed by Heath and Meidner (loc. cit.) was modified in an attempt to obtain a more rigid control of the magnitude of water-strain imposed on the leaves.

The Γ-values of leaves whose bases were supplied with water were first determined with a water-vapour deficit of 12 mm. Hg. in the circulating air-stream and at a leaf temperature of 30° C. The water was then replaced by a mannitol solution and the increase in the CO_2-concentration of the system observed over about 2 hours. The water-vapour deficit of the circulating air-stream was then reduced to zero and a steady Γ-value was obtained in a short time—this was considered to be the Γ-value of the leaf under the water-strain produced by the particular mannitol solution. The leaf bases were now cut under water and immersed in fresh water every 5 minutes for about 40 minutes. The water-vapour deficit of the airstream remained at zero and the CO_2-concentration was observed to decrease gradually until it reached a value near the original value for this particular leaf when supplied with water. The water-vapour deficit of the system was now adjusted to 12 mm. Hg. and the Γ-value could be read after a short time.

In order to produce degrees of water-strain in the palm leaves comparable to those imposed on wheat leaves by Heath and Meidner, the concentration of the guard-cell sap of palm-leaf stomata was determined plasmolytically. The guard cells of palm-leaf stomata underwent incipient plasmolysis when bathed in a mannitol solution of about 0·98 molar concentration. Epidermal cells were more severely plasmolysed in such solutions, but it was not possible to determine the stage of incipient plasmolysis for epidermal cells. A 0·7 M. mannitol solution was considered to bring about a degree of water-strain in palm leaves comparable to that which a 0·4 M. mannitol solution would produce in wheat leaves, whose guard cells underwent incipient plasmolysis when bathed in 0·68 M. mannitol solution.

The light intensity to be used with palm leaves was arrived at experimentally. At 1,400 f.c. palm leaves established a convenient level of Γ-value in a convenient time. Four leaves were used in the experiment and treatments were in the order: water—mannitol—water. Each leaf was used on three occasions so

that a total of 36 readings was obtained. The leaf temperature was kept constant at 30° C.

RESULTS

1. The results of the temperature treatments are shown in Fig. 1, in which log Γ has been plotted against the four temperature treatments. The change in

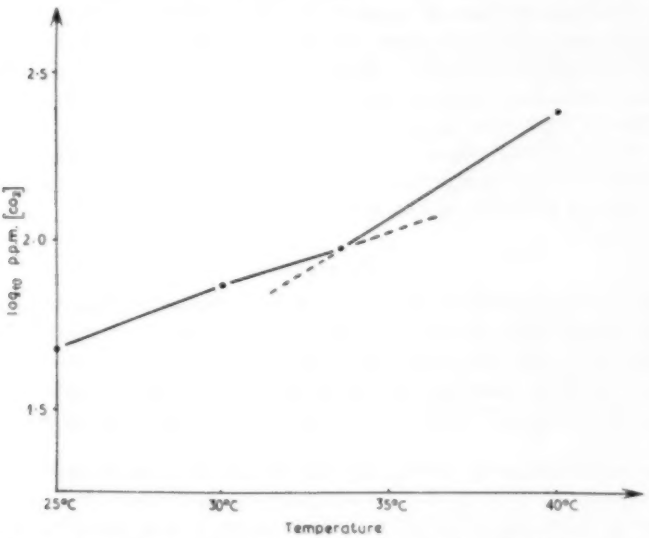

FIG. 1. Changes in the minimum intercellular-space carbon-dioxide concentration of leaves of *Phoenix reclinata* with temperature.
Light intensity: 1,400 f.c., WVPD: 12 mm. Hg.

TABLE I

Mean values (in parts per million) of minimum intercellular-space carbon-dioxide concentrations (Γ) of four leaves of Phoenix reclinata *supplied with water and 0·7 molar mannitol solution at 30° C. and under an illumination of 1,400 f.c.*

Treatments	*Mean Γ-values*
Water	78·5
Manitol	122·1
Water	79·2

Significant difference for $P = 0{\cdot}001$, 5·1;
for $P = 0{\cdot}05$, 2·8.

slope at 33° C. is of the same order of magnitude as that found by Heath and Orchard (loc. cit.) for leaves of onion and of coffee.

2. The results of the water-strain treatments with palm leaves are summarized in Table I, in which the mean Γ-values are shown for leaves first

supplied with water, then with mannitol solution, and finally again with water following the mannitol treatment.

The difference between the mean Γ-values of leaves supplied with water and those of leaves under water-strain are significant at $P < 0{\cdot}001$. The difference between the mean Γ-values measured before the mannitol treatment and those obtained after the mannitol treatment did not reach significance even at $P < 0{\cdot}05$. There were seven occasions when the Γ-value following the mannitol treatment was slightly lower than that obtained before the mannitol treatment and there were five occasions when it was slightly higher than before the mannitol treatment. The fact that the mean value for water treatment following mannitol treatment shown in Table I is slightly higher than the original value for water treatment is due to a single occasion when the second Γ-value remained 12 p.p.m. higher than the first, almost certainly owing to incomplete outward diffusion of mannitol solution.

DISCUSSION

The results quoted above show that leaves of *Phoenix reclinata* respond both to high temperature and to water-strain with a sudden and marked increase in their minimum intercellular-space carbon-dioxide concentration. In this species, therefore, the observed phenomenon of midday closure of stomata may be due to either of these two factors, or both, interacting, as indicated by Rees.

The results also show that in the palm leaves used there would appear to have been no after-effect of experimentally imposed water-strain as was discussed by Heath and Meidner for wheat leaves. It may be added here that *Phoenix reclinata* leaves are fairly stiff and fibrous, with comparatively large conducting strands. One may presume, therefore, that in this leaf outward diffusion of mannitol solution could occur largely unhindered by mechanical obstacles.

Attention may be drawn to the fact that during the time of measuring Γ-values of leaves supplied with water, the water-vapour pressure deficit of the circulating airstream amounted to 12 mm. Hg., whereas for leaves treated with mannitol solution the WVPD of the airstream during the time of measuring Γ-values was zero. Experiments during which Γ-values of detached palm leaves supplied with water were measured at a WVPD of 12 mm. Hg. as well as at zero WVPD showed that if such leaves suffered different degrees of water-strain, on account of different transpiration rates, these water-strains must have been small and were not reflected in the Γ-values measured.

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The Development of Mitochondria in *Arum* Spadix

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WITH FIVE PLATES AND ONE FIGURE IN THE TEXT

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SUMMARY

Electron micrographs of *Arum* spadix cells prepared at four stages of development all showed abundant sections of mitochondria with tubular ingrowths (microvilli). At the earliest stage (when the spadix cells were still dividing) there was an average of 9 sections of microvilli per mitochondrion. In later stages of development, when the cells were growing by elongation, the number of microvilli rose to 22.

It was reported earlier that the succinoxidase activity of the mitochondria of *Arum* spadix increased as the spadix developed, and it is now shown that the rise in enzyme activity parallels the increase in length of microvilli.

INTRODUCTION

DURING the last decade the ultrastructure of mitochondria has been extensively investigated. Many reports describe the characteristic double-membrane system bounding these cytoplasmic organelles and it is now generally accepted that invaginations of the inner bounding membrane into the body of the mitochondrion give rise to the internal membrane system (Palade, 1953).

It was recognized at an early stage that mitochondria from different tissues differed both in the number of ingrowths per mitochondrion and in the shape of these ingrowths; this is manifest in the numerous descriptive terms used—cristae, tubuli, sacculi, microvilli. These observations, coupled with the knowledge that many powerful enzymes are located in the mitochondria, have led to speculation about the relationship between mitochondrial structure and metabolic activity. Possible connexions between structure and function are most profitably sought in a tissue in which the mitochondria undergo change during development. Such a tissue is provided by the spadix of *Arum*. Earlier work with mitochondria from *Arum* spadix (Simon, 1959) has shown that the oxidase enzymes of the mitochondria become more active as the tissue ages. In the work described in the present paper we have examined the ultrastructure of the spadix mitochondria at

comparable stages in the growth process. The results have been further correlated with observations on the growth of the spadix itself to determine whether mitochondrial development is in any way geared to growth by cell division or to growth by cell expansion.

MATERIALS AND METHODS

The distal, club-shaped part of the spadix of *Arum maculatum* is composed of a central core of tissue with large cells and very conspicuous air spaces surrounded by a densely packed cortical tissue of relatively small cells. This cortical tissue was used in the work described here.

Electron microscopy. Small portions of cortical tissue were embedded on two occasions. The first was in 1958, at Queen's University, Belfast, using material collected locally (Figs. 2, 4, 5, and 6), and the second in 1960 when some earlier stages of development were embedded from plants collected in Cheshire the previous year and transplanted to the Manchester University Experimental Grounds (Fig. 3). The tissue was fixed in the solution recommended by Palade (1952*a*) with the addition of 0·3 M. sucrose, and embedded in methacrylate. A preliminary examination of thin sections of the blocks was made by Mr. R. S. M. Revell of A.E.I., Manchester, with an EM6 microscope; Fig. 6 comes from this work. The remainder of the micrographs were made with a Siemens Elmiskop I using sections cut to a thickness of 0·025–0·035 μ with a Huxley ultra-microtome and stained for 1–2 hours in saturated uranyl acetate at 40° C.

Cell development. Inflorescences were collected at intervals during the season, at first from the transplants and later (from mid-March) from a population in Cheshire. The terminal part of the spadix was excised and weighed; a weighed portion of cortical tissue was then transferred to chromic acid for counts of cell number (according to Brown and Rickless, 1949) and measurements of cell size (taken as the mean of about 20 diameters). In the early stages of development, when the cortex was still small, portions of the central core of tissue were sometimes included in the block taken for maceration; the core cells were easily recognized under the microscope by their large size and could then be ignored. The total number of cells in the spadix was calculated by multiplying the number of cells per gramme in the sample of cortex by the weight of the whole spadix. This is of course an approximation, since it takes no account of the fact that the cells of the central core of tissue make some contribution to the weight of the spadix; but we do not feel that the error is serious enough to affect the argument that follows.

RESULTS

Development of spadix tissue. The first signs of the development of the spadix can be discerned in the buds of tubers lifted 9–10 months before flowering. The inflorescence develops underground until about the middle of April and then comes to full maturity above ground.

Spadices were collected at intervals over a period of 10 weeks starting in late February for counts of cell number and measurements of cell size. During this time the spadix increased in weight by more than one hundred times (Fig. 1). Its growth clearly falls into two phases: in the first, growth is characterized by cell division, and in the second growth is by cell enlarge-

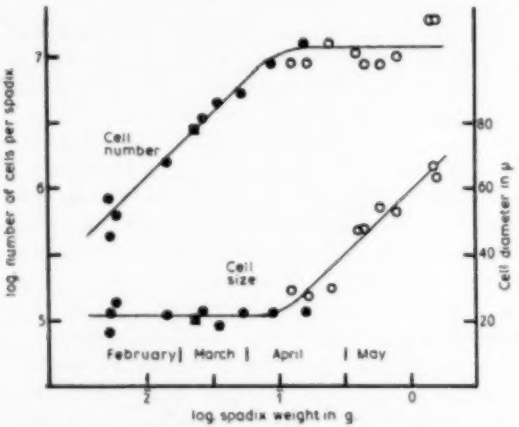

FIG. 1. Development of *Arum* spadix. Cell number and cell size are plotted as a function of spadix weight. The dark circles indicate that the spadix was taken from an inflorescence that was still below ground, the open circles showing stages above ground. The dark square indicates the stage I from which the micrograph of Fig. 3 was prepared.

ment. The change-over occurs in mid-April; it is significant that this is also the time when the inflorescence stalk elongates (presumably by cell elongation) to bring the inflorescence above ground.

Development of mitochondria. Electron micrographs were prepared at four stages of spadix development (Figs. 2–6 show typical fields in each case); at the first or Initial stage (I) the inflorescence is still below ground, while β, γ and ϵ are stages in the subsequent development above ground. Some details of spadix development at the first stage are given in Fig. 1, and drawings of the whole inflorescence at β, γ and ϵ can be found in the paper of James and Beevers (1950).

At each stage the micrographs show cells with abundant mitochondria (Fig. 2). The mitochondria are mostly circular or oval in outline, but a few are very elongate, as for instance in Fig. 5. The outlines show much variation in size (ranging from 0·2 μ to 2 μ); but this is only to be expected, because the dimensions of a thin section of a mitochondrion will depend on the angle and plane of sectioning. The modal diameter (the diameter most frequently encountered) can be taken as an estimate of mitochondrial size. At stages I, β and γ the modal diameter is about 0·4–0·5 μ, and at ϵ it is about 0·8 μ.

The sections are bounded by the typical double-membrane structure described for other mitochondria and consisting of two electron-dense layers about 5 mμ wide and separated by a less dense layer 7 mμ wide. Some of the sections of mitochondria show points at which the inner membrane is invaginated to form a projection towards the centre of the section (arrows in Figs. 4 and 6). These structures are significantly different from the 'cristae mitochondriales' described by Palade (1952*b*, 1953) in mammalian cells, since cristae are in the nature of flat internal ridges, their appearance in section depending markedly on the angle at which they are cut; the projections in *Arum* mitochondria seem, on the contrary, to be radially symmetrical, their appearance in section being confined to circles, ovals, and long narrow structures. We therefore refer to them, following Sedar and Porter (1955), as microvilli. In several of the sections the microvilli seem to be dilated at their distal end. Sitte (1958), who made similar observations in mitochondria from pea root meristem, concluded that the microvilli were in fact sack-shaped—and should therefore be termed 'sacculi mitochondriales'. However, it must be recognized that some sections of curving cylindrical microvilli would give the same dilated appearance.

Comparison of Figs. 3 to 6 suggests that in the early stages of development there are few microvilli, and that these are largely congregated around the periphery of the mitochondria. At stage ϵ the micrographs show numerous sections of microvilli, now no longer confined to the periphery. In an attempt to put this rather subjective impression on a quantitative basis, thirty micrographs were scanned and a count made of the number of sections of microvilli seen in each mitochondrion. The results (Table I) indicate that the number

TABLE I

Mitochondrial Development

Stage	Number of sections of microvilli per mitochondrion: Mean	S.E. of mean	No. of mitochondria counted	Succinoxidase. μ 1·O_2/hr./mg. mitochondrial nitrogen
I	8·9	±0·9	34	—
β	12·4	±1·1	41	2,750
γ	14·5	±0·7	41	3,500
ϵ	22·4	±2·0	19	5,250

of microvilli cut in a thin section of a mitochondrion has more than doubled between stages I and ϵ. It must be emphasized that what has been counted is the number of sections of microvilli seen within a thin section of a mitochondrion and it is necessary now to consider how this number relates to the total number and length of microvilli actually present per mitochondrion.

The thickness of the sections used for electron microscopy (about 0·03 μ) is such that a single section will include only a small part of a complete

mitochondrion (0·4–0·8 μ in diameter). The number of sections of microvilli seen in a mitochondrion will depend on the number of microvilli present and also on their individual lengths, for long ones are more likely to be included in a thin section than are short ones. The number of sections of microvilli is thus a measure of the total length of microvilli in the mitochondrion. We may therefore conclude here that between stages I and ϵ the total length of microvilli has roughly doubled, although we cannot say how far this is due to the elongation of existing microvilli and how far to the development of new ones.

DISCUSSION

Growth of microvilli and growth of cells. The electron microscope study of *Arum* mitochondria shows that the length of the microvilli increases as development proceeds and we may consider first the relation between this growth and the growth of cells. It will be recalled that at stage I the spadix is still growing by cell division, but at β, γ and ϵ growth is by cell enlargement (Fig. 1). It is clear from Table I that most of the growth of microvilli takes place at stages β, γ and ϵ—that is to say during the phase of cell enlargement. A similar conclusion was reached by Buvat and his colleagues (Buvat and Lance, 1958; Buvat, 1958; Lance, 1958; Caporali, 1959) working with the apical regions of stems of *Chrysanthemum* and *Elodea* and of roots of *Lens*. In each case they found that the mitochondria of meristematic cells had less internal structure than mitochondria from more mature cells.

Growth of microvilli and enzyme activity. It has long been known that *Arum* spadix has a rapid respiration and that at maturity the oxidation processes are so fast that the spadix becomes warm, but it is not sufficient to relate mitochondrial structure to tissue respiration—what we need is a comparison of the structure of mitochondria with the activity of their enzymes. Fortunately, mitochondria from *Arum* spadix have now been isolated (Bendall, 1958; Simon, 1959) and it is clear that the activity of oxidase enzymes increases as the spadix ages. More specifically, there is an increase in the rate of oxidation of succinate per mg. mitochondrial protein. This could be taken to imply that some non-active protein has become an enzyme as the spadix has aged, i.e., that the enzyme has appeared without any increase in the total amount of protein present—but another interpretation is also possible.

It is well known that, when mitochondria are disrupted and then centrifuged, about half the protein of the mitochondria remains in the supernatant, the remainder being sedimented down in the membrane fraction (Hogeboom and Schneider, 1955). It is evident that the mitochondria must contain much protein besides the enzyme succinoxidase—there are the soluble proteins released when mitochondria are damaged, and the proteins that constitute the membranes. Lehninger (1959) envisages the membranes as walls on which assemblies of respiratory enzymes are spaced at regular intervals, so that they will contain some proteins which are purely structural in character

together with others that are catalytically active, like succinoxidase and the enzymes of oxidative phosphorylation. Consider now the consequence of the synthesis of fresh succinoxidase protein in the membranes. The synthesis would increase oxidase activity, but there would be only a relatively small rise in total mitochondrial protein; in other words, oxidase activity per mg. mitochondrial protein would rise. Hence the initial biochemical observations would be accommodated by an hypothesis based on the idea that fresh succinoxidase is being synthesized on the membranes. Now if, as Lehninger (loc. cit.) suggests, the enzymes are spaced out on the mitochondrial membranes, the synthesis of fresh enzyme might well require the synthesis of additional membrane surface—and it was indeed this very possibility, that the amount of enzyme activity might be related to the amount of mitochondrial membrane, that gave impetus to the present investigation.

The results of the two studies, biochemical and structural, are brought together in Table I and it can be said at once that there is a parallel increase in the length of microvilli per mitochondrion and in succinoxidase activity per mg. mitochondrial protein, both quantities being roughly doubled between stages β and ϵ. These figures on their own do not compel the conclusion that oxidase activity depends on the length of microvilli; only if the present results could be seen as typical of many others would this point be established. The only relevant observations that we have been able to trace are those of Hanson and his colleagues with maize and soybean seedlings (Lund, Vatter, and Hanson, 1958; Hanson, Vatter, Fisher, and Bils, 1959; Key, Hanson, and Bils, 1960). These observations all point in the same direction as our own results—towards a close relationship between mitochondrial structure and function.

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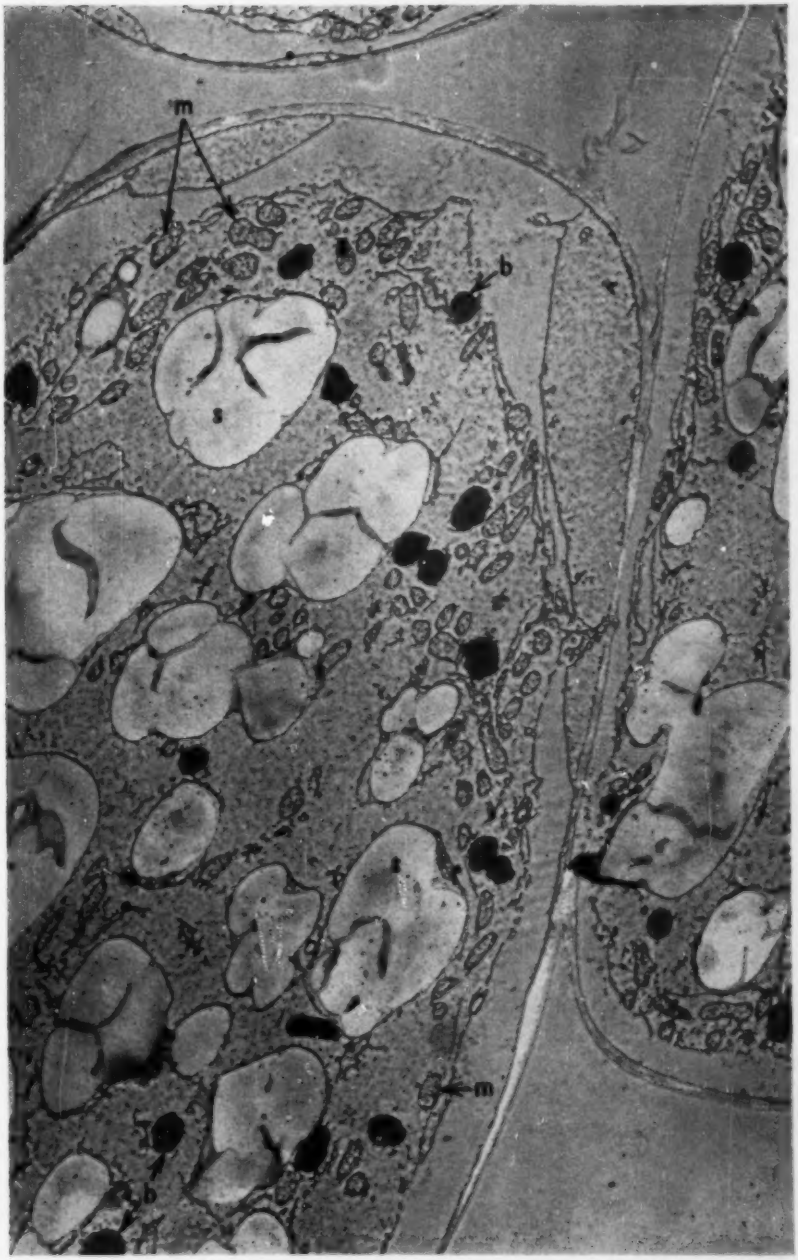

FIG. 2. Low-magnification electron micrograph of parts of three cells from an *Arum* spadix at stage *β*, showing starch grains (s), mitochondria (m), and dense, i.e. osmiophilic, bodies (b). ×6,000. Uranyl acetate stained.

E. W. SIMON AND J. A. CHAPMAN—PLATE I

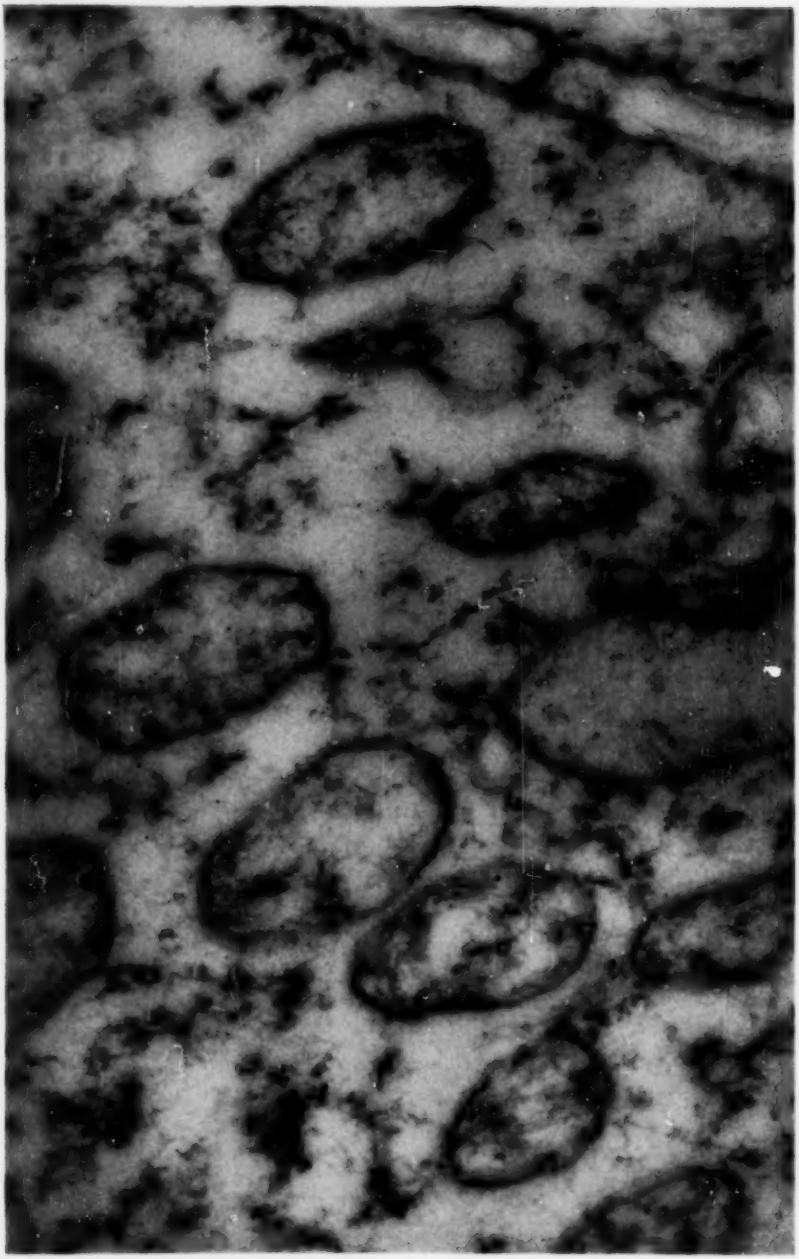

FIG. 3. Mitochondria from a spadix at stage 1 (first stage). A cell wall with a plasmodesma traverses the top right corner. Electron micrograph ×60,000. Uranyl acetate stained.

E. W. SIMON AND J. A. CHAPMAN—PLATE II

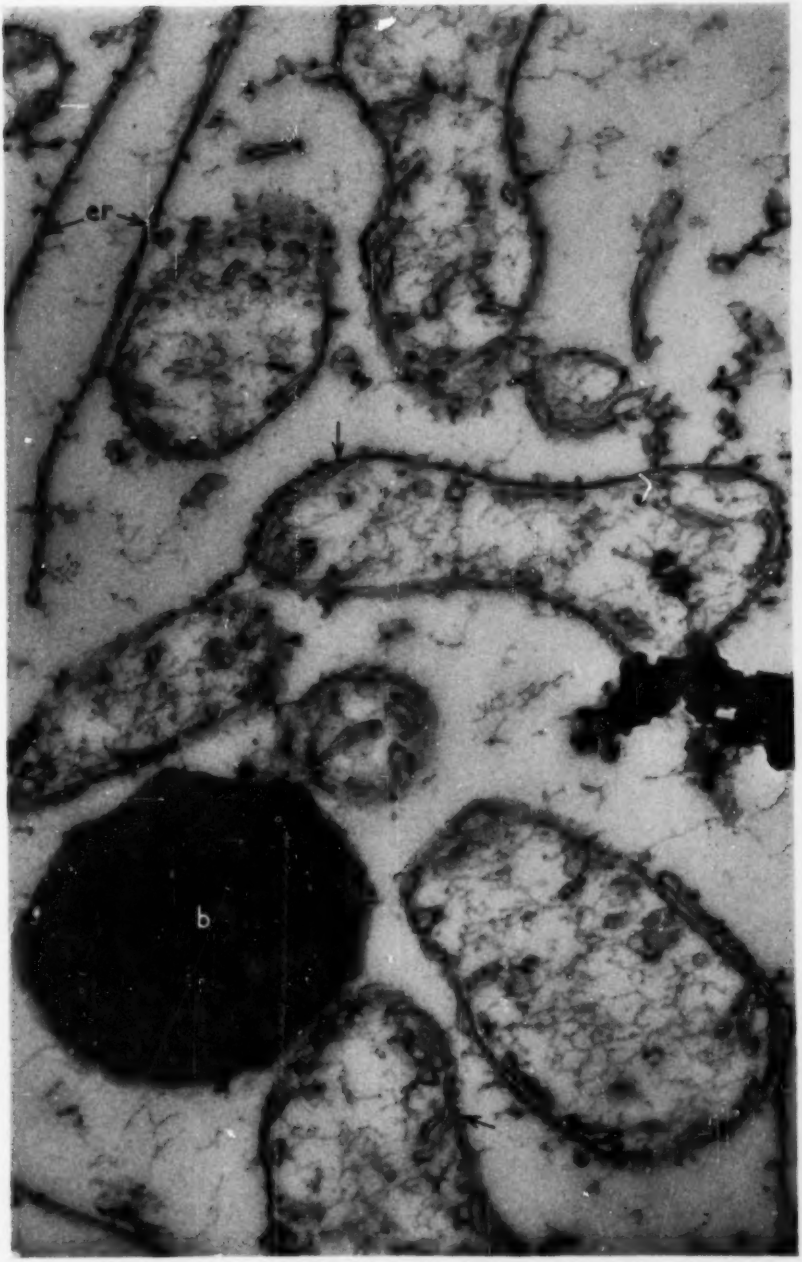

FIG. 4. Mitochondria at stage β (second stage). The field also includes endoplasmic reticulum (e.r.) and a dense body (b.). The arrows indicate invaginations of the inner limiting membrane of the mitochondria to form microvilli. Electron micrograph $\times 60{,}000$. Uranyl acetate stained.

E. W. SIMON AND J. A. CHAPMAN—PLATE III

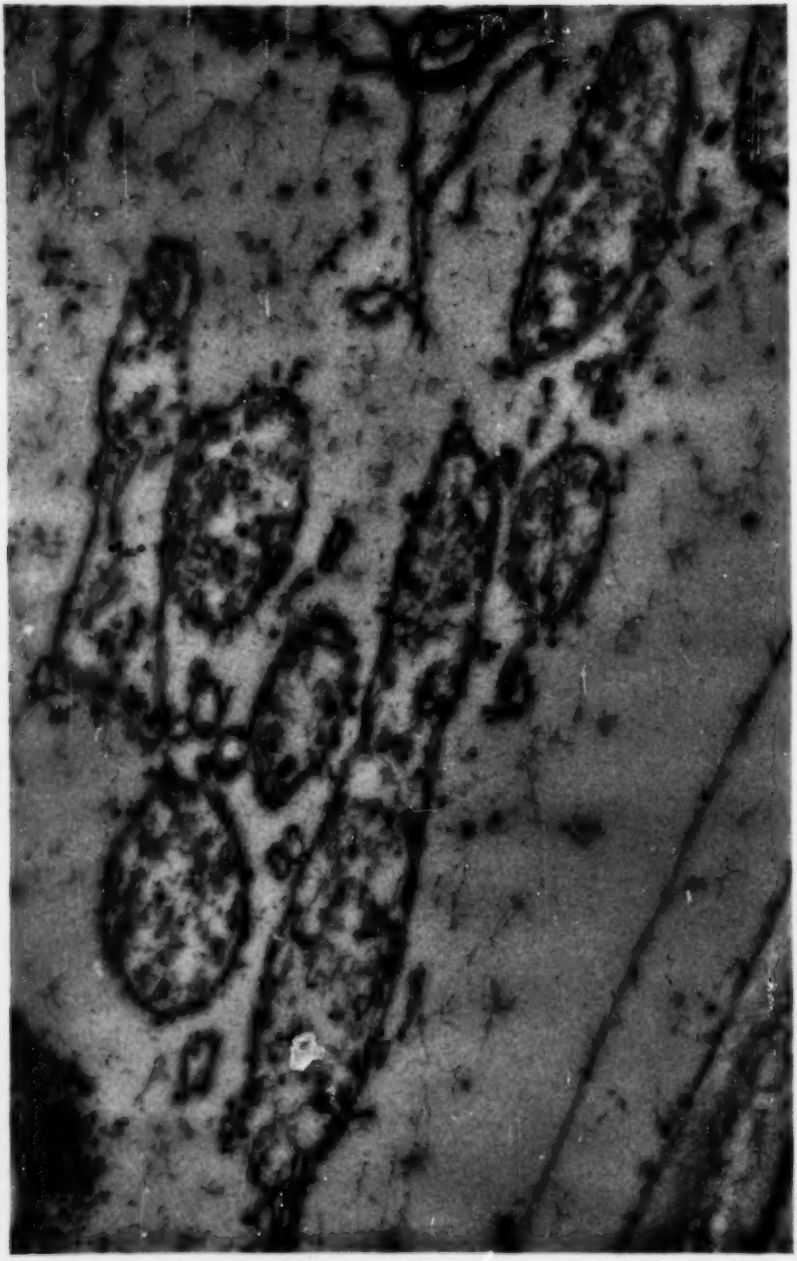

FIG. 5. Mitochondria at stage γ (third stage). Electron micrograph ×60,000. Uranyl acetate stained.

E. W. SIMON AND J. A. CHAPMAN—PLATE IV

FIG. 6. Mitochondria at stage *e* (fourth stage). The arrow shows an invagination of the mitochondrial membrane. Electron micrograph × 55,000.

E. W. SIMON AND J. A. CHAPMAN—PLATE V

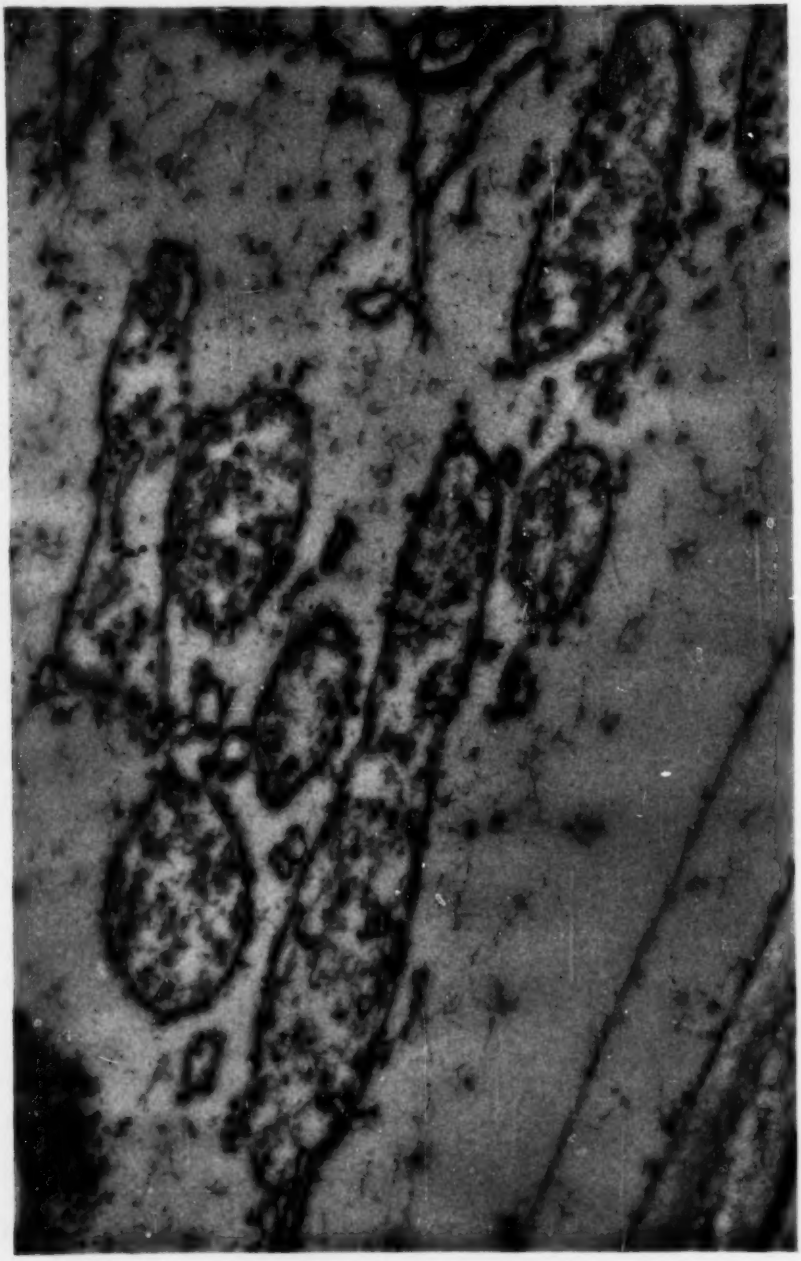

FIG. 5. Mitochondria at stage γ (third stage). Electron micrograph ×60,000. Uranyl acetate stained.

E. W. SIMON AND J. A. CHAPMAN—PLATE IV

FIG. 6. Mitochondria at stage ε (fourth stage). The arrow shows an invagination of the mitochondrial membrane. Electron micrograph × 55,000.

E. W. SIMON AND J. A. CHAPMAN—PLATE V

Some Problems of Mitochondrial Growth

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WITH TWELVE PLATES (CONTAINING 21 FIGURES) AND ONE FIGURE IN THE TEXT

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SUMMARY

Observations on mitochondrial growth are recorded from the meristem of a liverwort (*Anthoceros laevis*) and from a Chrysophycean flagellate (*Chrysochromulina brevifilum*). No sign of *de novo* origin has been obtained in either case. In both the commonest form of mitochondrial reproduction is from growth leading to clusters of mutually attached mitochondria which are presumed to separate later. Some stages in the formation of clusters are illustrated and some of the processes involved analysed in a preliminary way. Mitochondrial growth is seen to be a somewhat more complicated total process than has sometimes been thought.

INTRODUCTION

THOUGH from certain points of view mitochondria rank among the better-known cytoplasmic organelles both in plants and in animals, there are many aspects of their structure which have scarcely been studied at all. One of these is their growth. In my own experience there are only two basic facts known to me about this, namely (*a*) the well-known demonstrations with cine-photography and phase contrast on living cells (mostly animal) that mitochondria are flexible and mobile with a capacity to break up and (perhaps less certainly) to coalesce, and (*b*) the more recent demonstration with the electron microscope that in some very small pigmented flagellates, notably the two known species of the genus *Micromonas* (Manton and Parke, 1960; Manton, 1959), in which only one mitochondrion is present in each cell, this mitochondrion divides to keep pace with cell division, bending into a U-shape first. In this genus, therefore, there is the strongest reason for believing that mitochondria are derived from pre-existing ones and do not normally arise *de novo*. In other types of cell with more numerous mitochondria this is less easily demonstrated though commonly pre-supposed, but in no case known to me has a serious attempt been made to trace the structural details involved.

The observations to be recorded here will not completely fill this gap. Mitochondrial growth is manifestly a complicated process with several components. The appearances that these collectively produce vary greatly at different times and in different parts of one and the same organism. Interpretation of even common appearances is not always easy and in detail may be impossible without more information than can at once be extracted from fixed material. Some appearances are nevertheless so striking that they

immediately attract attention even when encountered in a new type of organism and it is therefore virtually certain that fundamental principles of general applicability will eventually emerge, in the light of which the various individual observations recorded here will become more accurately describable. In the meantime it is a sufficient objective to draw attention to some of the more salient features which can be encountered.

MATERIAL AND METHODS

The principal material to be quoted is the meristem of *Anthoceros laevis* as used previously (Manton, 1960, 1961). Most of this was fixed locally near Leeds in 1958 using standard osmic fixation (for details see Manton, 1960). This has, however, been supplemented to a limited extent by material from Cornwall treated in other ways in the autumn of 1960. Potassium permanganate fixation had been attempted in 1958 and 1959 but without success. For some reason the schedule first introduced by Luft (1956) is most unsatisfactory when applied to this particular material and only by increasing the strength and temperature of the fixative and substantially reducing the fixation time were useful though still imperfect results obtained. The three micrographs reproduced as Figs. 2, 6, and 7 were from an embryo received by post in November 1960 and fixed in 5 per cent. $KMnO_4$ in distilled water without buffer and used at room temperature for ½ hour. The remaining micrographs are selected from nearly 4,000, accumulated for many purposes from the 1958 fixations.

The material of the flagellate *Chrysochromulina brevifilum* was fixed by the standard procedures now current in Leeds for work carried out in collaboration with the Marine Biological Station at Plymouth. The culture is the holotype of the species (No. 39 in the Plymouth collection) described by Parke, Manton, and Clarke (1955) and kindly supplied by Dr. M. Parke, whom I wish to thank.

I have also gratefully to acknowledge financial assistance towards the cost of the flagellate work from the Development Commission. Thanks are also due to the technical staff of the Botany Department at Leeds, notably Miss Sheila Wright for help in preparing the work for publication.

OBSERVATIONS ON THE MERISTEM OF *ANTHOCEROS*

In a young embryo, as, for example, in the unvacuolated telophase cell from the archesporium illustrated in Plate VIII of Manton 1961, mitochondria are scattered in the peripheral cytoplasm, where they commonly yield circular or only slightly anisodiametric profiles with an average diameter of ½ to 1μ. At other times they are cylindrical, as in the metaphase cell illustrated in Plates IV and V of the same paper, in sections cut parallel to the spindle. At the other extreme, cells which have experienced considerable extension growth, or spore mother cells which have rounded up and reached their full size though not yet undergone meiosis, commonly have mainly cylindrical mitochondria or at least give profiles which suggest this.

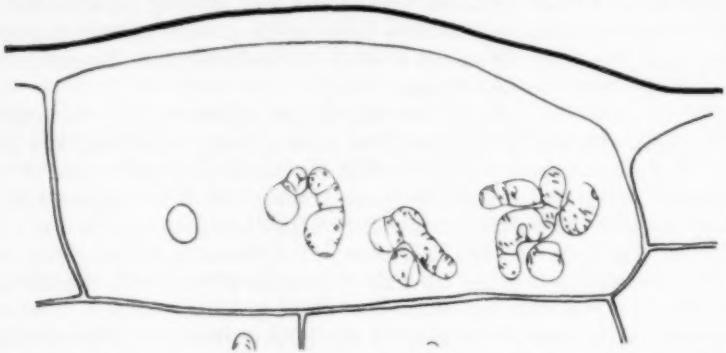
Fig. 1 is a good example of an apparently cylindrical mitochondrion from a full-sized spore mother cell in a condition of full metabolic activity uncomplicated by manifestations of division. The mitochondrion lies along the surface of a starch-filled chloroplast and is surrounded by cytoplasm crowded with other inclusions that need not at present concern us. Within it the compound wall and numerous internal villi (invaginations from the inner wall layers into the lumen of the organ) are conspicuous. The contents of villi are somewhat distended, a detail which may or may not involve some minor osmotic distortion during osmic fixation. The large number and relatively uniform size of these villi are nevertheless characteristic of mitochondria in this position in contrast to those from more rapidly growing parts of the vegetative tissue, in which villi cannot so easily (in this material) be demonstrated, though they can of course be found.

The reason for this difficulty is at once apparent from Fig. 2, which represents a somewhat similar mitochondrion from a young vacuolating wall cell, fixed in potassium permanganate. This has failed to preserve any of the cytoplasm except the membrane systems which are in consequence very clearly revealed. They include mitochondria, part of a golgi body in face view (bottom), fragments of tubes, and parts of the plasmalemma in a very imperfect condition near to the cell wall. Within the mitochondria the villi are less distended and relatively smaller than in the specimen of Fig. 1. It is not easy to assess the extent or direction in which the contents of villi can become distorted by post-mortem changes. I am therefore not prepared to express an opinion as to which, if either, of these two images corresponds the more closely to conditions in life. The relative shortness of the villi in Fig. 2 together with the admixture of longer ones resembling the cristae of animals is nevertheless undoubtedly accurate, and an indication that the specimen belongs to a rapidly growing cell. It suggests that the mitochondrion is immature and growing by some process of intussusception of wall material from which a continual formation of new microvilli is taking place. Whether the larger cristae are merely older or are potentially transecting the whole organ as part of a division process is unknown. It is easy to suggest that in such a specimen mitochondrial cleavage if it occurred might be expected to be transverse. Direct information about this is, however, extremely difficult to obtain.

Transverse cleavage of a cylinder is, however, by no means the only possible mode of growth and indeed it may turn out to be a rather special type, only found under a fairly restricted range of circumstances and perhaps less frequently than the appearances in sections at first suggest. Plates II and III illustrate other manifestations of growth from cells in the early stages of vacuolation. Fig. 4 shows a cell from the archesporium which will almost certainly differentiate into an elater and not a mother cell. It is not yet very large, though vacuoles are conspicuous. The mitochondria are, however, not scattered evenly through the cytoplasm but are present in clusters, three of which are included in this section. Figs. 3 and 5 represent two different sections through a cluster from a similar cell. In Fig. 3 the union of all the

profiles present is unmistakable. In Fig. 5 this is less obvious and it is clear that without the use of serial sections it may be impossible to know whether profiles of mitochondria encountered side by side in a section at any one level are in fact from separate individuals or not.

The text-figure illustrates another example of clusters *in situ*, this time from an epidermal cell cut close to one of the lateral walls in a longitudinal section. The field includes only the peripheral protoplasm in which several clusters are evenly scattered. It is again necessary to have more than one section to know whether within a cluster the component profiles are attached



Drawing traced over a micrograph to show the distribution of mitochondrial clusters along a wall face in an epidermal cell exposed almost tangentially in a longitudinal section of a young capsule (long axis of the capsule across the page). A micrograph taken from another section through the right hand cluster of this cell is reproduced Plate V, Fig. 6. Magnification of the diagram approximately *c.* 7,000.

at other levels or not. This evidence is provided for the right-hand terminal cluster of the diagram by Fig. 6, which shows the same field in another section in which all parts of the cluster are unmistakably united. This preparation is fixed with potassium permanganate and the details of the villi and cristae are again instructive. Some cristae appear branched or strongly curved (see especially the level of the drawing) and curvature can be apparent even in very small villi.

All this suggests that clusters are the result of growth and not of fusion and that in some way the shape of the invaginations may be important. A branched or curved partition, even if incomplete, can delimit a lenticular surface from which independent growth might start. The very narrow points of contact between some members of a cluster might therefore express the actual size of the area from which a bulge began. Small circumscribed surfaces are common objects among growing mitochondria (e.g. Fig. 7) but this interpretation of their significance implies a mode of growth quite other than simple partitioning or constriction of a branched or cylindrical organ.

One further point should be noted, namely the fact that if, as is very probable at least in some stages of cell growth, a mitochondrial cluster of the type

illustrated in the diagram were somewhat flattened within the layer of cytoplasm lining the cell wall, a section cut perpendicularly to the plane of flattening might only be distinguishable from that of a layer of independent spherical or cylindrical mitochondria by quite small differences, such as those distinguishing Fig. 7 from Fig. 2. The precise interpretation of the details of single sections is thus by no means as simple a matter as might at first have been thought.

Manifestations of mitochondrial growth of a different type are contained in Plates IV–VIII. The first shows a low-power general view of the lining layer of the capsule wall cut in tangential longitudinal section with the long axis of the capsule obliquely vertical. Mitoses have not yet ceased and the edge of a telophase plate just enters the field in the right-hand top corner from an adjacent cell belonging to the same layer. In the row depicted, each cell is shorter than wide, giving further proof of recent active cell division and for anyone interested it might be an instructive comparison to look at the anaphase cell illustrated in Manton 1960 which comes from the same tissue and was reproduced at the same magnification as that of Plate IV. As in that case, vacuoles are conspicuous though extension growth is slight. The cytoplasm is dominated by the large chloroplast and contains many scattered mitochondria, some of which in each cell are in the peculiar condition which is about to be described. Other cytoplasmic components which need not concern us here are fat bodies, golgi bodies, and various tubes and granules, the latter probably not completely preserved.

Interpretation of Plate IV will perhaps be easier by comparison with Plate V, which represents part of the middle region of the same field more highly magnified. On either side of the end wall transecting the field is a greatly extended mitochondrion, beside others of more normal shape. The extended mitochondria at first sight might be described as of dumb-bell shape in a condition suggesting the plastic deformation of a cylinder brought about by pulling apart of the ends. This description would, however, undoubtedly be wrong. These mitochondria are not cylinders being pulled apart but disks with thin centres and thicker rims which are becoming bent into a bowl or saucer shape. This is made abundantly clear by many specimens which have been encountered cut in a plane perpendicular to that of Plate V. One is indeed included above the cell plate in the top right-hand corner of Plate IV although at this low magnification it may not be clearly recognizable. Plates VI and VII, reproduced at successively higher magnification, will, however, exemplify this. The curious ring-shaped profiles, each with a compound wall on both surfaces, are clearly sections through the central hollow parts of bowls comparable with the upper specimen in Plate V though cut transversely. The material circumscribed by the ring is thus not mitochondrial contents but cytoplasm complete with the usual granular or tubular inclusions.

That there is no difference in thickness of the mitochondrial wall between the rim and either of the surfaces of the bowl or disk is made clear by Fig. 12. This contains parts of both rim and centre at a magnification sufficient to resolve

the details of the compound wall. This specimen, it should perhaps be noted, is from the archesporium, and the cytoplasm is, in consequence, more densely crowded with contents (granules, tubes, and young vacuoles) than in the other cells illustrated.

There is no special tissue for which disk-shaped mitochondria are characteristic. They can be found in all layers of the capsule wall and at several ages of spore mother-cells. Their frequency nevertheless varies considerably from capsule to capsule. This makes it difficult to ascribe any function to them with certainty though it is easy to suggest that they might be manifestations of specially active growth in which increase in the contents of the lumen has not kept pace with increase of intercalary wall material. If this is true they would be expected to be temporary and unstable conditions which will pass over into other configurations, such as those of a new cluster, if cleavage or branching were to set in. It is not necessary to assume that all clusters must have arisen in this way, but that such disks are one means of rapidly increasing mitochondrial growth-centres is suggested strongly by cognate observations on another type of organism which will be considered in the next section.

OBSERVATIONS ON *CHRYSOCHROMULINA BREVIFILUM*

This small marine flagellate was described and the external morphology illustrated in Parke *et al.*, 1955. The internal structure, which is shown here for the first time, is not essentially different from that of other simple Chrysophyceae, notably several species of the genus *Chrysochromulina* already placed on record in Parke, Manton, and Clarke (1959) and Manton and Leedale (1961), except for the clarity with which certain growth processes have been encountered and which make it relevant to the present discussion. To understand these it is sufficient to know that the organism is a naked biflagellate cell with the external appendages attached posteriorly relative to the prevailing direction of swimming and that it contains two chromatophores, a nucleus, a so-called leucosin vesicle, and the usual minor protoplasmic inclusions. The cytoplasm can also contain food vacuoles, since this species, like most others of the genus, is phagotrophic. The body in life is covered by large numbers of small translucent scales, and possesses the characteristic filamentous appendage to which the name haptonema has been given. None of the last three characters will concern us in interpreting the micrographs presented here but anyone wishing for information about them will find it in the paper cited (loc. cit., 1955).

The reason for including this organism will at once be apparent from a glance at Plate IX. Here, in the central cytoplasm lying between the nucleus and two plastids, is an elongated mitochondrion expanded at one end in a manner exactly recalling the curved disks of growing *Anthoceros*. The general topography of the cell from which this detail is taken can be obtained from Plate X in which Figs. 14*a* and 14*b* represent at a low magnification two significantly different sections through it. Figs. 13 to 14*b* collectively show that the

disk is near to the non-flagellar pole and that it occupies only a relatively small part of the whole mitochondrion.

Other views of mitochondrial disks of various shapes and cut in different planes are contained in Figs. 18*a* to 19 with low-power views of the cells from which they came in Figs. 16 and 17. The first (Fig. 16) is an oblique transverse section through a cell containing a resting nucleus but the second (Fig. 17) is a mitotic metaphase with parts of some chromosomes visible in the centre. This section includes two chromatophores and the leucosin vacuole and it is therefore near to the non-flagellar pole. The exact level in the cell from which Fig. 16 is taken is not determinable.

I have series of several sections through each of these cells but space only permits reproduction of three samples at a higher magnification. Figs. 18 *a* and *b* represent two views (*m*) of the disk of Fig. 17 cut in a plane closely comparable with that of Plate V above, though with differences of detail becoming apparent in Fig. 18*b*. Fig. 19, which is taken from another section of the series of Fig. 16, shows a disk cut approximately at right angles to this. The interpretation is identical with those of the comparable sections of *Anthoceros* (Plates V–VII).

Examples of what I believe to be later stages in mitochondrial growth in this organism are included in Fig. 15 and in the final plate. The cell of Fig. 15 contains a ring-shaped mitochondrion, otherwise structurally normal, while a somewhat similar cell with a U-shaped mitochondrion is represented by Fig. 20*a*. In both these specimens the centre of the area delimited by the ring or U marks the nearest point to the nucleus, and in both specimens the level of section is not far from the cell surface. Sample sections cut at deeper levels through each cell are reproduced in Fig. 21 and in Figs. 20 *a* to *c* respectively. The gradual break-up of the ring or U towards the interior of the cell can be traced in detail, and though space does not permit publication of the whole series in either case there is no break in the continuity between all the mitochondrial profiles encountered at the deepest level with the ring or U nearer the surface. Contrary to first appearances therefore it seems probable that all or most of the mitochondria present in an entire cell of this species may in the end prove to be a connected branch system or at least to have originated from one.

Two further facts support the suggestion that a disk in this species is a normal part of mitochondrial reproduction. In my experience disks when encountered occur singly within a cell. It is of course difficult, from negative evidence, to exclude the possibility that had longer series been available, another disk might have been reached in some of the cells illustrated. In random sections I have, however, never so far met with one which even suggested the presence of two disks transected simultaneously. The weight of positive evidence at present therefore favours the interpretation that mitochondrial disks form one at a time and that they do so in a definite position in the cell namely beside a plastid and near to the non-flagellar pole. That they are likely to appear in some definite time-relation with the mitotic cycle is

probable, though to establish this would require more knowledge of mitosis in this material than we have at present. The detection of a well-developed disk in a metaphase cell is nevertheless perhaps no accident.

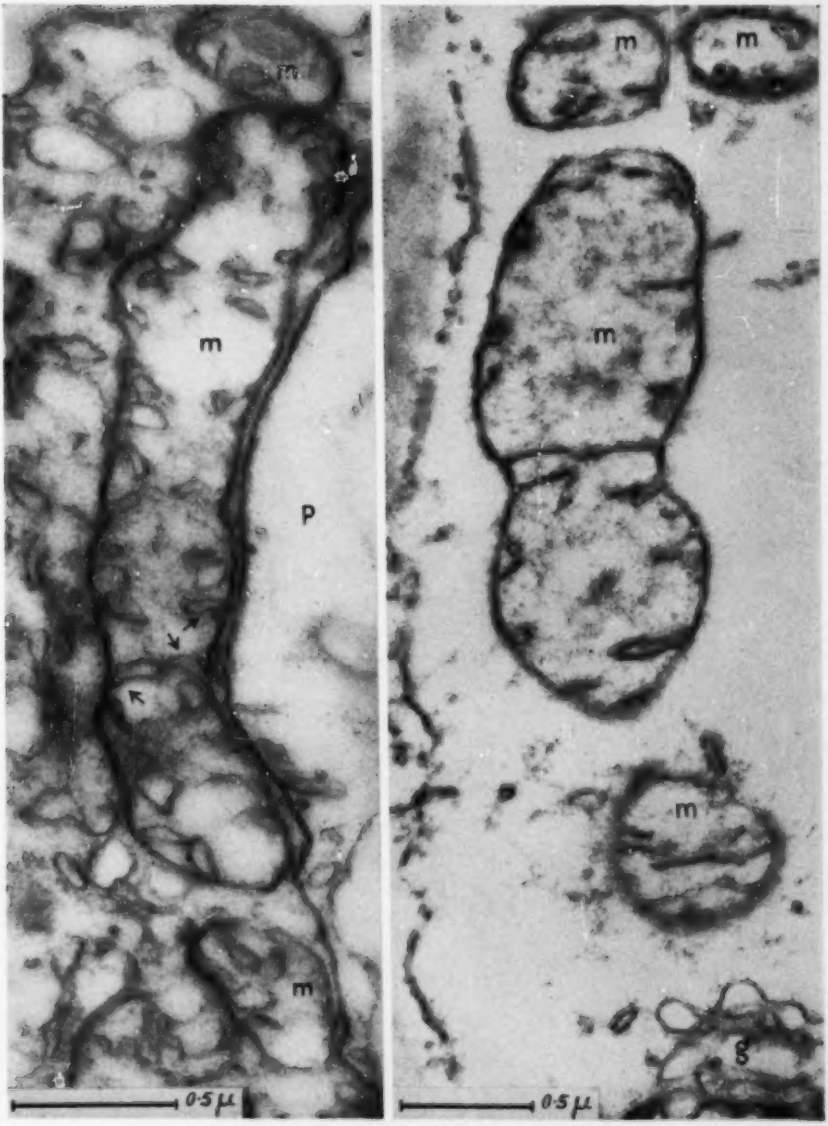
DISCUSSION

It should perhaps be emphasized that *C. brevifilum* is the first species of *Chrysochromulina*, among nine which have now been investigated by means of sections, in which mitochondrial growth of the type just described has been detected. It is also the first species in which any stage of mitosis has been recognizable as such. It is therefore not yet certain whether the details illustrated are peculiar to this species or whether they could have been encountered in others had the cultures been fixed at an appropriate stage. The resemblance to *Anthoceros* is a strong reason for believing that *C. brevifilum* cannot be unique. On the other hand it is equally unnecessary to assume that the details of mitochondrial growth will be uniform everywhere and there is indeed already sufficient evidence that this is not so. Apart from the special case of *Micromonas* (p. 421 above) in which the cells are so small that a disk could scarcely have failed to be detected had mitochondrial division normally involved one, the marked difference in the relative frequency of disks among different embryos in *Anthoceros* suggests that this is perhaps a special feature associated with particular conditions, e.g. high growth-rate.

The general interpretation that I would put upon these observations is therefore to suggest that while the details of mitochondrial growth and replication are likely to vary, perhaps within wide limits, in different types or conditions of cell, those so far investigated are all consistent with an origin of new mitochondria by descent from pre-existing ones and not by *de novo* synthesis. They are also all consistent with the idea that there is a fundamental similarity in the basic growth-pattern in spite of morphological differences which at first sight seem spectacular. Such differences nevertheless might prove to be fairly simply explained by quite small divergences in the time sequence of the various components of the process as a whole. Of these there are undoubtedly five, namely (*a*) increase of wall substance, (*b*) increase of the contents of the lumen, (*c*) formation of new villi, (*d*) delimitation (by means of cristae or in other ways) of independently growing surface areas, (*d*) separation of the products of growth.

No evidence has so far been obtained about the mechanisms involved in (*d*). The physical and chemical factors involved in the others are equally unknown. It is nevertheless not difficult to appreciate the potential importance of quantitative differences in the incidence of these various individual processes on the morphology of the product.

It is therefore perhaps legitimate in the present state of knowledge to regard the diverse appearances so far encountered as indicators of local metabolic or other differences within cells rather than of fundamental differences within their mitochondria. This being said it is, however, obvious that mitochondrial



1 2

FIG. 1. Elongated mitochondrion near a starch-filled plastid in a rounded spore mother cell of *Anthoceros laevis*. Osmic fixation, micrograph H6641, × 50,000.

FIG. 2. Elongated mitochondrion near an end wall of a rapidly growing young vegetative cell from the capsule wall fixed in potassium permanganate. In addition to the mitochondria the membrane systems preserved include part of a golgi body in face view (*g*), parts of the plasmalemma near the cell wall (left) and some fragments of cytoplasmic tubes. Micrograph B763, × 40,000.

I. MANTON—PLATE I

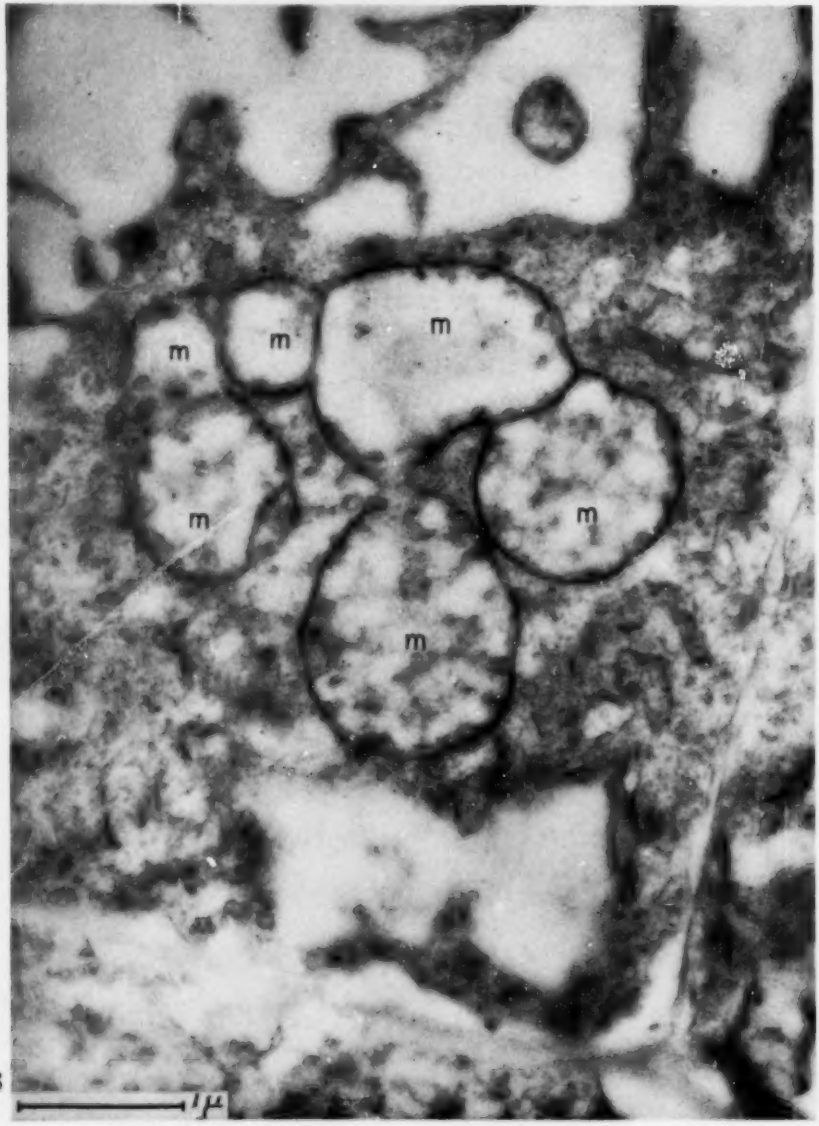
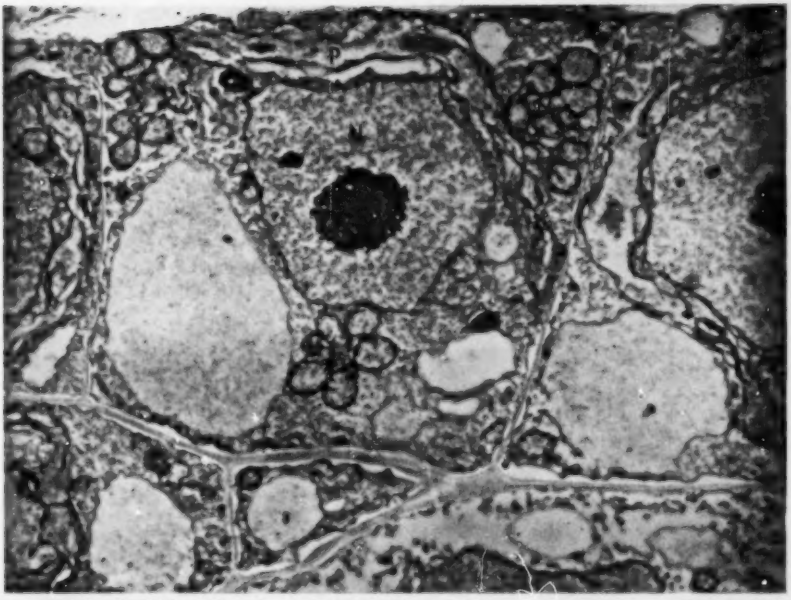


FIG. 3. Part of a cluster of mitochondria near an end wall of a cell from the archesporium of *Anthoceros* fixed in osmium tetroxide. Micrograph H7900, × 25,000.



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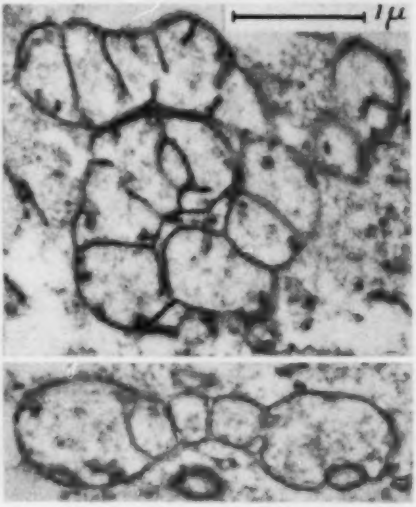

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FIG. 4. Low power view of the archesporium of *Anthoceros* in the region of a young vacuolating elater, to show distribution of mitochondrial clusters in it. Micrograph H7887, ×*c.* 6000.

FIG. 5. Another section of the cell of Fig. 3 to show a different view of what is in fact a coherent mitochondrial cluster. Micrograph H7874, ×12,000.

FIG. 6. A mitochondrial cluster from the epidermal cell shown in the text-fig. (p. 424) from another section through the right-hand end cluster. Permanganate fixation. Micrograph B963 ×25,000.

FIG. 7. Part of what may be a mitochondrial cluster cut perpendicularly to a wall. Permanganate fixation. Micrograph B785 ×30,000.

8

FIG. 8. Tangential section through the innermost layer of the capsule wall of *Anthoceros* (long axis of the capsule obliquely vertical) to show the general field from which Fig. 9 is taken. Osmic fixation. Micrograph H7287, ×*c.* 6000.

I. MANTON—PLATE IV

9

FIG. 9. Part of the centre of the field of Fig. 8 to show extended mitochondria in parts of two adjacent cells. Osmic fixation. Micrograph H7286, × 15,000.

10

FIG. 10. Part of a subepidermal cell in an early stage of extension growth showing a transverse section of a bowl-shaped disk-mitochondrion (arrows) beside a normal one. Other organelles include a golgi body (*g*), a fat body (*F*), vacuoles, granules, and rough tubules of the endoplasmic reticulum. Osmic fixation. Micrograph H7258 × 30,000.

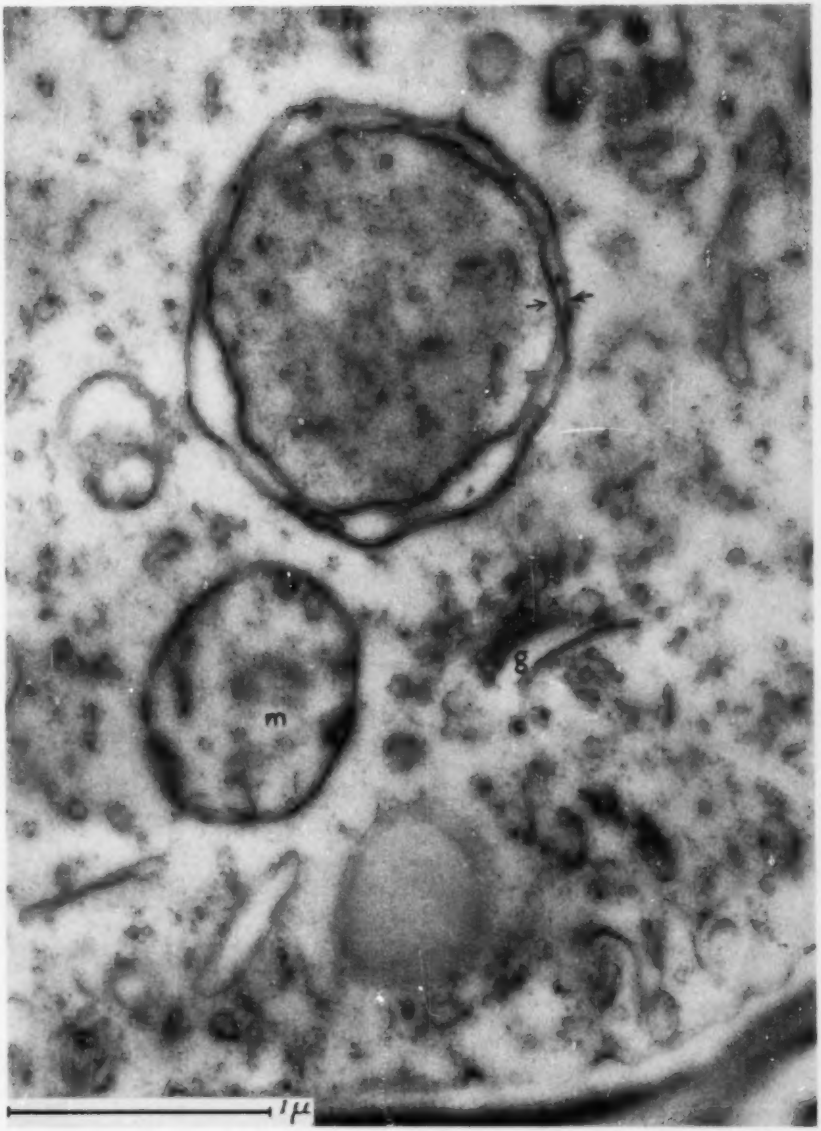

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FIG. 11. A more highly magnified transverse section of a bowl-shaped mitochondrial disk from another specimen. Osmic fixation. Micrograph H7423, ×40,000.

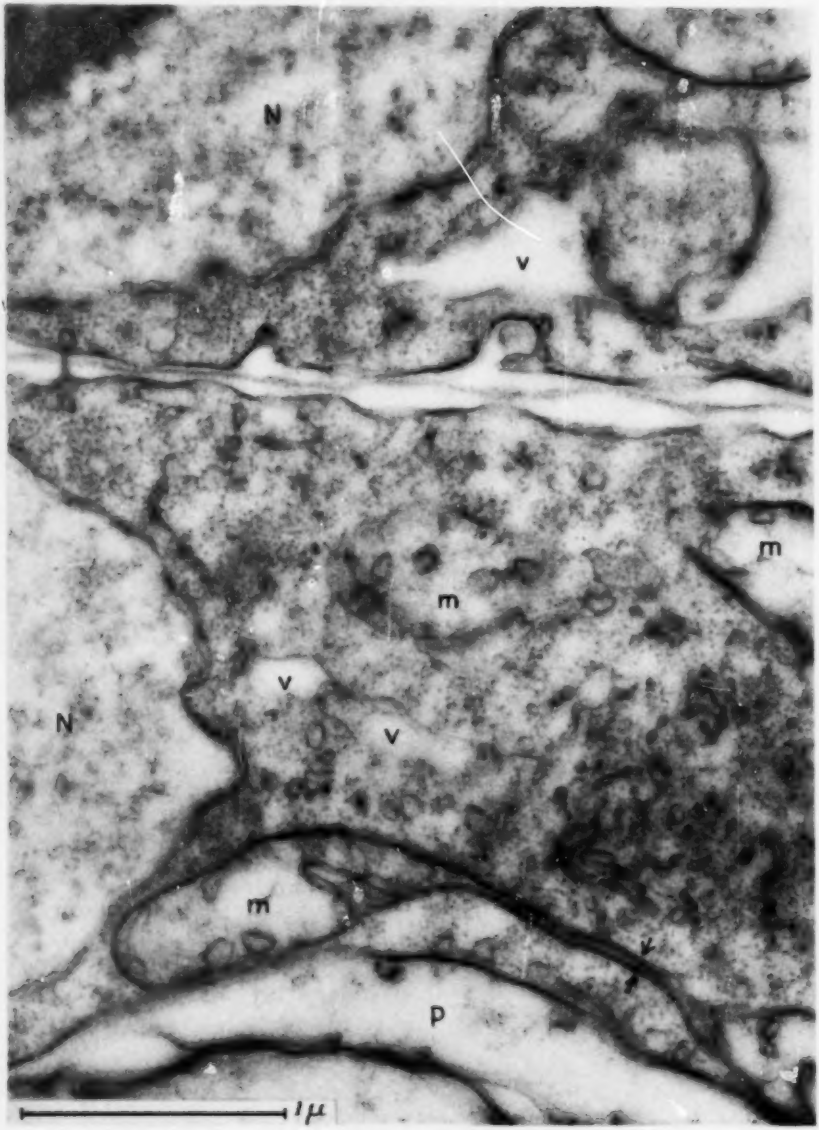

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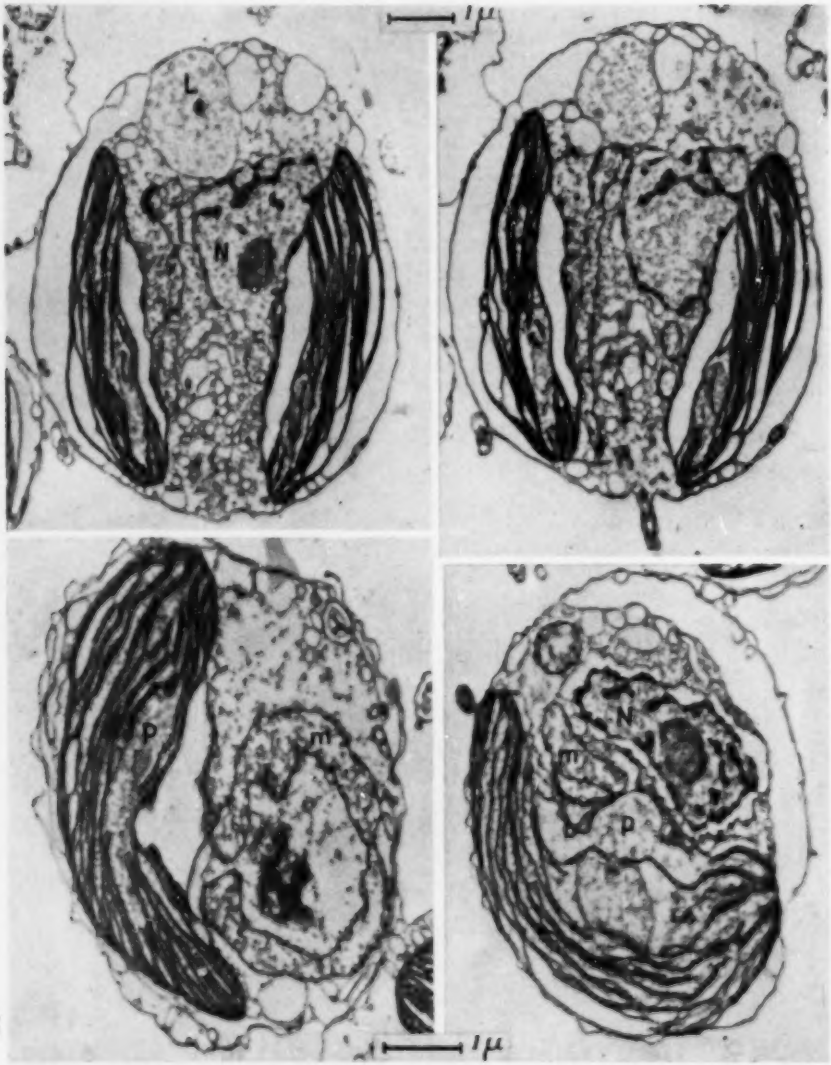
FIG. 12. Part of a young embryonic archesporial cell showing parts of two nuclei (*N*), some very young vacuoles (*v*), many cytoplasmic granules and tubes, and an extended mitochondrion showing the compound wall structure. Osmic fixation. Micrograph H3382, ×40,000.

13

Chrysochromulina brevifilum

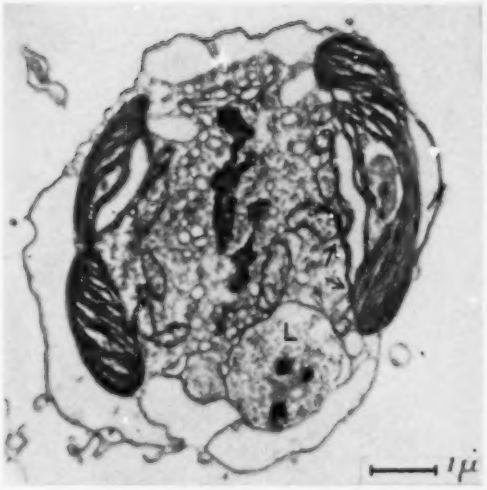
FIG. 13. Part of the centre of the cell of Figs. 14*a* and 14*b* showing an 'extended' mitochondrion (arrows) beside the nucleus (*N*), and leucosin vesicle (*L*), the inner surfaces of the two plastids (*P*) visible laterally. Micrograph B1492 ×40,000.



FIGS. 14*a* and 14*b*. Two different sections through the cell of Fig. 13 to show the general topography. Micrographs B1500 and B1501, × 10,000.

FIG. 15. Part of a cell showing a ring-shaped mitochondrion; another section from a deeper level in the same cell reproduced as Fig. 21. Micrograph B1502, × 12,000.

FIG. 16. A cell with an 'extended' mitochondrion (*m*) shown at a higher magnification in Fig. 19, other letters as in Pl. IX. Micrograph B362, × 12,000.



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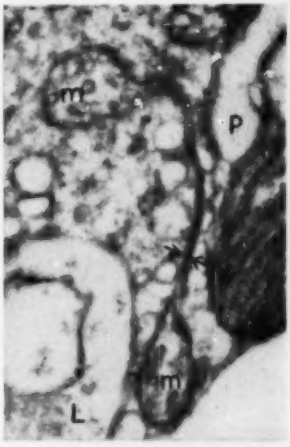
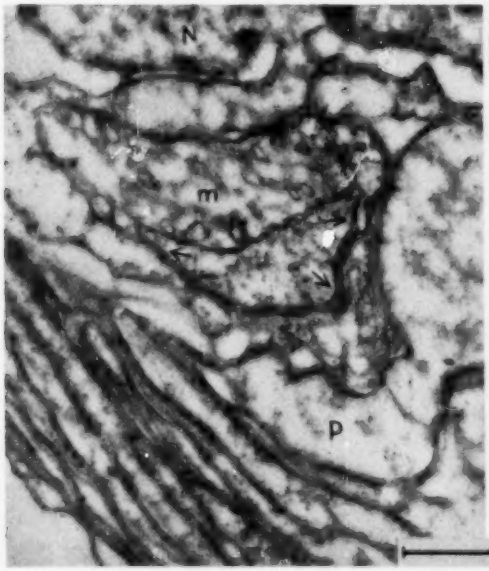


18*a*



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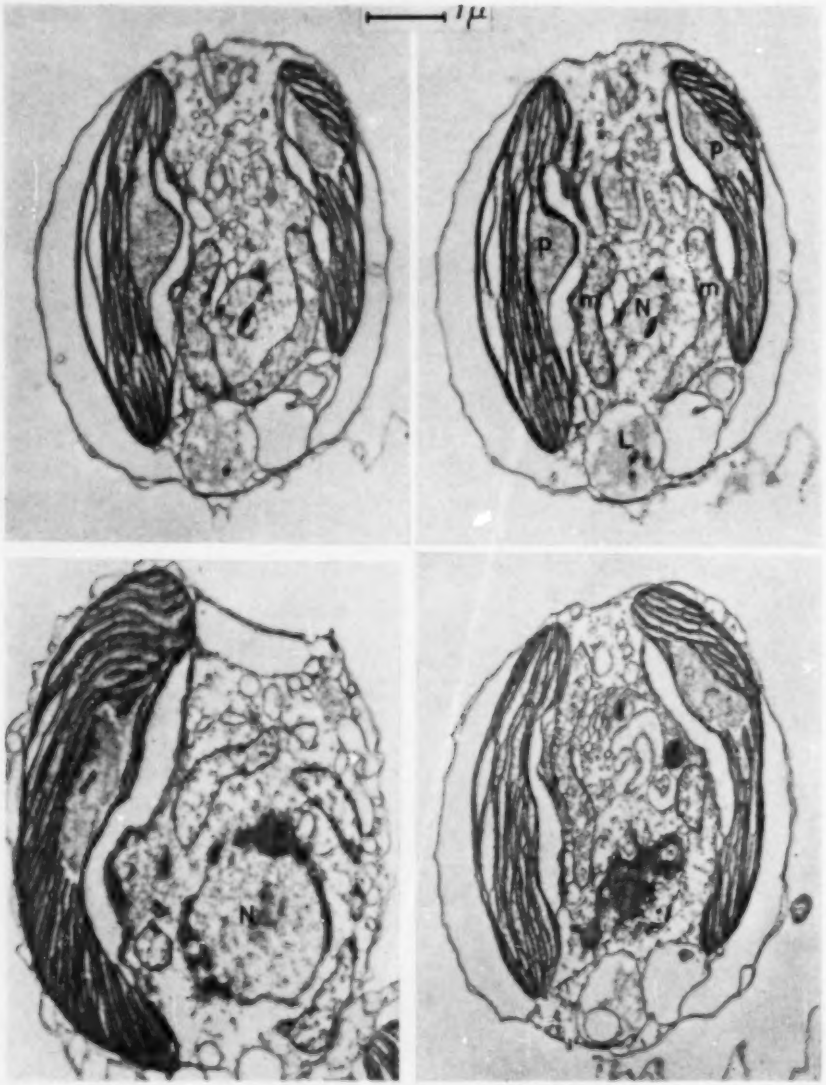
18*b*

FIG. 17. A cell in metaphase. Detail from the bottom right-hand side reproduced at a higher magnification in Fig 18*b*. Micrograph B404 × 10,000.

FIGS. 18*a* and 18*b*. Two different levels through the extended mitochondrion (arrows) of Fig. 17. Letters as in Plate IX. Micrographs B397 and B405. Fig. 18*a* × 30,000, Fig. 18*b* × 40,000.

FIG. 19. More highly magnified view of the mitochondrial disk (arrows) from the cell of Fig. 16 in another section. Micrograph B365 × 40,000.



20*a* 20*b*

21 20*c*

FIGS. 20 *a–c*. Three different levels through a cell showing gradual break-up of a U-shaped mitochondrion near the cell surface into finger-like mitochondria at deeper levels. Letters as in Plate IX. Micrographs B1504, B1505 and B1508, × 12,000.

FIG. 21. Another section of the cell of Fig. 15 at a much deeper level showing numerous mitochondrial fingers surrounding the nucleus but all ultimately connected with the ring seen in Fig. 15. Micrograph B1503, × 12,000.

growth and replication is a far more complicated process than might at first have been thought and that a very great deal about it still remains to be determined.

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Dormancy in Rice Seed

II. THE INFLUENCE OF COVERING STRUCTURES

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WITH FIVE FIGURES IN THE TEXT

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SUMMARY

Most of the dormancy in rice seed can be accounted for by the inhibitory influence of the husk, and most of the residual dormancy after dehusking can be attributed to the inhibitory influence of other covering structures—either the pericarp or testa, or both. It is shown that the rate of water absorption is the same in dormant and non-dormant seeds and that dormant seeds are capable of absorbing sufficient water for germination. The covering structures therefore do not cause dormancy by restricting the entry of water.

Removal of a small area of the husk breaks the dormancy of a large proportion of the seeds; but for some seeds this treatment is ineffective whereas removal of the entire husk would break dormancy. The site of the excision of a small area of the husk can alter the effectiveness of the treatment: removal of a portion of husk immediately over the embryo is no more effective than excising a similar portion nearby, but the removal of part of the husk some distance from the embryo is not as effective. Sealing the perforations with paraffin wax has little effect except when carried out as soon as possible after the excision is made, and then only in positions distant from the embryo.

Attempts to extract a water-soluble or ether-soluble germination inhibitor from the husk and other parts of dormant seed or to demonstrate the presence of inhibitors by indirect methods have not been successful. Nor has it been found possible to extract a water-soluble germination stimulator from seed which has broken dormancy.

The implications of these results are discussed.

INTRODUCTION

IT is well-established that seed dormancy in many species is caused by the inhibitory influence of structures covering the embryo rather than by some factor within the embryo itself (Toole *et al.*, 1956). The inhibitory influence of various seed-covering structures has been recognized for some considerable time: it is generally believed that in cereals Hiltner (1901) was the first to demonstrate this phenomenon. Since then the inhibitory effect has been demonstrated in most of the more common cereals. The earlier work on cereals has been discussed in some detail by Harrington (1923); more recently Caldwell (1959) has briefly surveyed our present knowledge of the problem. Harrington was probably the first to suggest some of the more likely ways

in which the covering structures might inhibit germination. He suggested that they may prevent the following: '(1) diffusion of an inhibiting substance outwards, (2) entry of water, (3) increase in respiration or alteration in its nature by [not] allowing ready exchange of oxygen and respiratory products, (4) a more obscure "stimulus" of the living protoplasm.' The fourth suggestion is perhaps too vague to be of value, but the first three possibilities are useful in suggesting an experimental approach. Although these hypotheses were formulated almost forty years ago, in general the mechanism by which the covering structures impose dormancy in cereal seed is still by no means clear. There are, however, exceptions to this generalization. Elliot and Leopold (1953) have shown that the inhibitory influence of the oat husk can be attributed to the presence of a water-soluble germination inhibitor in the husk. But even in this species the picture of dormancy is not complete because either the pericarp or testa or both are also responsible for a considerable proportion of the dormancy (Atwood, 1914) and the mechanism of this component of dormancy has not been completely elucidated. Nevertheless, the work of Elliot and Leopold has stressed the importance in any study of the dormancy of cereal seeds of considering the possibility of inhibiting substances in the covering structures. More recently Black (1959) has published results on *Avena fatua* (wild oats), indicating that inhibitors in the caryopsis itself may be important in controlling dormancy.

Harrington thought that seed dormancy in cereals could be explained entirely on the basis of the inhibitory influence of covering structures: he suggested that '. . . the embryo of most cereals is never essentially dormant, dormancy being imposed by coat structures'. But since then evidence has been published which supports the view that true embryo dormancy exists in some cases, e.g., in wild oats (Black and Naylor, 1959) and in barley (Pollock, 1959).

It was with these ideas as a starting-point that the present investigations into covering structures in rice seed were started.

MATERIAL AND METHODS

One variety was used for all experiments: this was Toma 112 (*Oryza sativa*, L., sub-species *indica*), a pure-line selection made from indigenous material at this station. 'Standard germination tests' were carried out as described in the first paper of this series (Roberts, 1961); occasional departures from this technique are noted in the text.

RESULTS

Assessment of the relative importance of covering structures in causing dormancy

The inhibitory influence of the lemmata and paleae in a number of cereals where these structures form part of the natural dispersal unit is well known. Recently, for example, their inhibitory effect has been demonstrated in oats (Schwendiman and Shands, 1943; Elliot and Leopold, 1953; Furste, 1958),

in wild oats (Naylor and Christie, 1957; Kommedhal *et al.*, 1958; Black, 1959), in barley (Pollock, 1959), and in rye (Furste, 1958). The first experiment was therefore designed to examine the extent to which the husk influences the germination of rice seed.

Freshly harvested seed was sun-dried to about 11 per cent. moisture content and then stored at ambient temperature (mean about 27° C.). Weekly standard germination tests were carried out starting 10 days after harvest using three treatments as follows: (1) seeds not dehusked (control); (2) all seeds

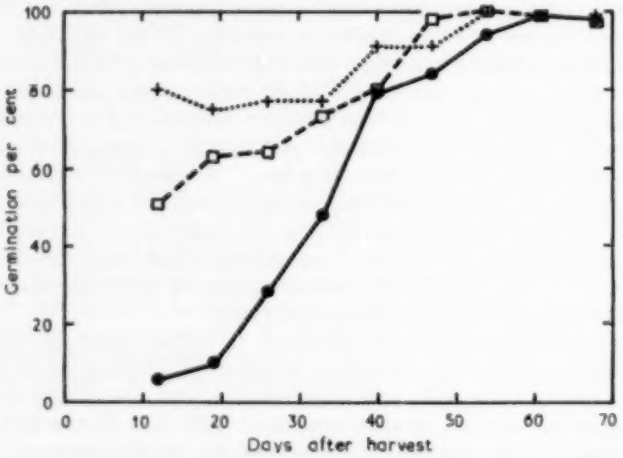

FIG. 1. The effect on dormancy of removing the husk. ●——● intact seed (control); □- - -□ seed dehusked 3 days after harvest; +·····+ seed dehusked immediately before each test.

dehusked 3 days after harvest; (3) seeds dehusked, but not until immediately before each germination test. Germination in this experiment was in general slightly higher in the light than in the dark but, in order to simplify the presentation, the results of the tests carried out in the light and the dark have been combined in the construction of Fig. 1. This figure shows that in most seeds the removal of the husk allows the germination of some seeds which would otherwise be dormant. But both treatments 2 and 3 demonstrate that removal of the husk does not affect all seeds: some of them remained dormant for the same length of time as the seeds on which the husk was retained so that maximum germination was reached at approximately the same time in all treatments. One curious and as yet unexplained feature of this experiment is that for a small proportion of the seeds dehusking immediately before each test was effective in removing dormancy, whereas dehusking soon after harvest was not; this effect was also confirmed in a later experiment.

From the present experiment it may be concluded that the most important

factor causing dormancy in rice seed is the inhibitory influence of the husk; but at least one other factor also exists. The question now arises as to whether a second factor preventing germination could be caused by other structures covering the embryo. There is evidence that such structures in other cereal seeds can also have an inhibitory influence on germination. For instance, in wheat—where the husk does not form part of the normal dispersal unit—it has been shown (Tung-Fang, 1957; Fitzgerald, 1959) that excision of the endosperm is effective in breaking dormancy, and Harrington (1923) showed earlier that removal of the coating structures with sulphuric acid had a beneficial effect. The observations of Wellington (1956) suggest that it is

TABLE I

Effect of structures external to the embryo on seed dormancy in rice

Treatment No.	Treatment	Per cent. germination		
		In the light	In the dark	Mean
1	Seed with husk intact (control)	12	4	8
2	Seed with husk, cut in half transversely	32	18	25
3	Dehusked seed	60	56	58
4	Dehusked seed, cut in half transversely	90	82	86
5	Dehusked seed; pericarp and testa scarified with fine sand-paper in the position of the centre of the palea	96	86	91

the pericarp of wheat which is important in controlling dormancy. Even more significant in relation to the present study, in barley—a cereal where the husk does form part of the dispersal unit—it has been demonstrated (Pollock *et al.*, 1955) that the main inhibitory structure is the pericarp. As already mentioned in the introduction, it is evident that either the pericarp or testa, in addition to the husk, is also important in oats.

In order to investigate the extent to which structures other than the husk impose dormancy in rice seed, a sample of seed was used which showed a particularly high proportion of residual dormancy after the husk was removed. This sample had been maintained in a dormant condition for 20 weeks after harvest by dry storage at $3° \pm 2°$ C. A standard germination test was carried out on 5 treatments. A description of these treatments together with the results are listed in Table I. An examination of this table shows that removal of a small area of the pericarp and testa by scarification (Treatment 5) results in the disappearance of dormancy from most of the seeds where the dormancy has not already been removed by dehusking. A further removal of the distal half of the endosperm and associated pericarp and testa does not cause any additional increase in germination; in fact, the results indicate that germination might be slightly inhibited by this treatment (Treatment 4 compared

with Treatment 5). Even when half the entire seed is removed, the remaining half of the husk covering the embryo still exerts considerable inhibitory influence (Treatment 2).

Water absorption by dormant seeds

One of the factors causing seed dormancy in some species is the impermeability of the seed coverings to water. This form of dormancy is particularly common, for instance, in *Leguminosae*, though it also occurs in a large number of other families (Crocker and Barton, 1953, p. 123). Hiltner (1901) attributed the prompt germination of dormant cereal seed when the outer coverings were broken to the rapid intake of water. In order to test this possibility in rice, an experiment was carried out to compare the water absorption of dormant and non-dormant seed.

After the normal sun-drying following harvest, one-half of a sample of seed was stored in an air-tight container in an air-conditioned room (mean temperature about 18° C.) and the other half was stored in a similar container in a refrigerator (3°±2° C.) for 5 months. Under these conditions most of the seed stored at the low temperature remained dormant, while all the seeds stored at the higher temperature broke dormancy. An apparatus was constructed in order to subject both samples of seed to the same constant high humidity at a constant temperature, viz., 100 per cent. R.H. and 35°±1° C. A diagram of this apparatus is shown in Fig. 2. Immediately before the seed was put in the apparatus a standard germination test and a moisture determination were carried out on the 'dormant' and 'non-dormant' seed. The results of the germination tests were as follows: 'dormant' 20 per cent. and 'non-dormant' 100 per cent. germination. During the course of the experiment, which lasted 88 hours, more seeds broke dormancy: a germination test carried out at the end of the experiment showed that the 'dormant' seed was then capable of 35 per cent. germination. During the course of the experiment small samples were removed from the 'dormant' and 'non-dormant' seed and moisture determinations were carried out in triplicate by the oven-drying method (ground sample at 105°±1° C. for 24 hours). The moisture contents are expressed as a percentage of the weight of the seed before drying.

The results of the experiment are given in Fig. 3. This graph shows the moisture content of both the 'dormant' and 'non-dormant' samples plotted against time. It will be seen that there is very little difference between the two curves except for the last four points. At this stage considerable germination was occurring in the 'non-dormant' sample—more than 50 per cent.—and thus the moisture determinations carried out on these last four points represent the moisture contents of a mixture of young seedlings as well as seeds. It is suggested that the inclusion of young expanding tissue would account for the differences in moisture contents shown between the 'dormant' and 'non-dormant' samples at this stage. Extra samples were taken at the same time as those taken for moisture determinations and were stored in miniature air-tight bottles for 7 days at 32° C. At the end of this period the seeds were

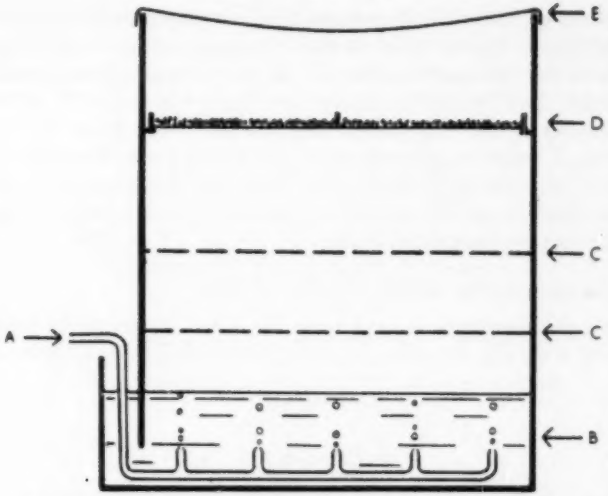

FIG. 2. Apparatus for increasing the moisture content of seed with humid air under standard conditions. Air is continuously blown in at A and is then humidified by passing through warm water (B) thermostatically controlled at 35°±1° C. The humidified air then passes upwards through two perforated metal screens (C) which prevent splash and then through the bottom of the seed tray (D) which is made of nylon netting. The tray is divided into two equal sized compartments so that dormant and non-dormant seed can be treated at the same time. The opening at the top of the apparatus is covered with a cotton cloth (E).

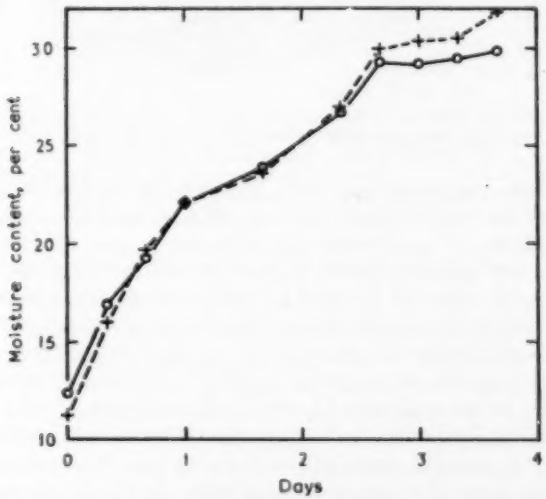

FIG. 3. The increase in moisture content with time of dormant (○——○) and non-dormant (+ - - -+) populations of seed treated in the apparatus shown in Fig. 2.

examined for germination. Even from those samples taken after 48 hours, i.e., at approximately 24 per cent. moisture content, 18 per cent. germination occurred in the 'dormant' sample and 24 per cent. germination occurred in the 'non-dormant' sample.

The results of this experiment indicate that there is no barrier to the absorption of water in dormant seeds and that the rate of absorption is the same as in non-dormant seeds. Dormant seeds are capable of absorbing sufficient water for germination. The reason for their inability to germinate must therefore be sought elsewhere.

Surgical experiments on the husk

When a rice caryopsis is shed from the plant, it retains its husk, which consists of a closely adhering and interlocking lemma and palea. Fig. 4 is

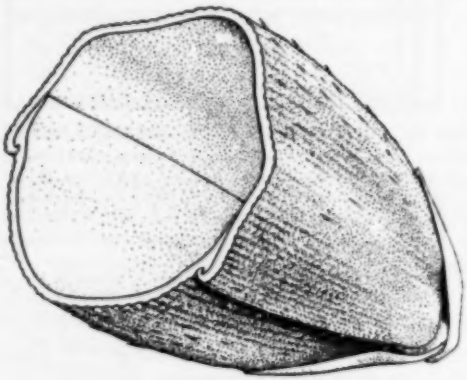

FIG. 4. Drawing of the husk of a rice seed cut in half transversely to show the morphology of the interlocking lemma and palea.

a diagram showing this arrangement. It is possible that the husk could form a barrier to the ready inward diffusion of a gas such as oxygen, or to the outward diffusion of some inhibiting substance, gaseous or otherwise. Caldwell (1959) has quoted evidence suggesting that the breaking of dormancy in some cereals might be due to the outer coverings of the seed being made permeable following attack by fungi. If the husk of rice does in fact prevent germination during the dormant period by acting as a barrier to the diffusion of some substance, then it should be possible to remove dormancy by excising a small part of the husk covering the embryo. Excising other parts of the husk would probably also have a similar effect. In addition, it might be possible to obtain more information about the nature of the mechanism by resealing the perforated part of the husk after varying lengths of time. These ideas suggested the following experiments.

Four sites were chosen for the removal of small areas of husk. These

positions are shown in Fig. 5. The excisions were made with a dissecting needle and care was taken to avoid damaging the underlying pericarp. An attempt was made to keep the size of the perforation as constant as possible: this resulted in the excised portion having an area of about 1 sq. mm. and

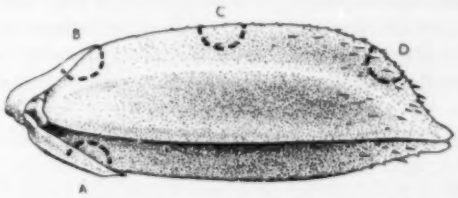

FIG. 5. Drawing of a rice grain showing the sites on the husk where excisions were made. A, proximal palea; B, proximal lemma (i.e. over embryo); C, median lemma; D, distal lemma.

TABLE II

Results of the first surgical experiment on rice husk

Treatment No.	Treatment	Per cent. germination
1	Husk intact (control)	8
2	Entire husk removed	66
3	Entire husk removed; paraffin placed over embryo	56
4	Proximal region of palea perforated	45
5	Proximal region of lemma perforated	40
6	Proximal region of lemma perforated; perforation sealed with paraffin after 72 hours	33
7	Median region of lemma perforated	46
8	Median region of lemma perforated; perforation sealed with paraffin after 5 seconds	20
9	Median region of lemma perforated; perforation sealed with paraffin after 72 hours	47
10	Distal region of lemma perforated	9
11	Husk intact; paraffin placed in median lemma position	0

never more than 2 sq. mm. In some of the treatments the place from which the portion of husk was removed was subsequently covered with paraffin wax having a low congealing point (54·5° C.). After treatment the seeds were subjected to standard germination tests. The design and results of the experiments are shown in tables. From the results of the first experiment (Table II) the following conclusions may be drawn. For most seeds the removal of a small part of the husk near to or covering the embryo is effectively the same as removal of the entire husk in breaking dormancy; but in a certain proportion of the seeds excision of a small part of the husk is ineffective, whereas removal of the entire husk breaks dormancy. Comparing the various positions of excision there is little difference in effectiveness between the three

positions nearest to the embryo, but excision in the position distal to the embryo was ineffective. The results of the treatments in which paraffin wax was used to seal the husk after excision are difficult to interpret with certainty as they are open to the objection that it is not known how effective the wax was as a sealing agent. Furthermore, it is possible that the wax itself had some slight inhibitory action. Nevertheless, there is an indication that sealing as soon as possible after the excision was made (i.e. after approximately 5 seconds) reduced the stimulatory effect of the perforation more than sealing after 72 hours.

TABLE III

Results of the second surgical experiment on rice husk

Treatment No.	Position of perforation	Period elapsed before sealing with paraffin	Per cent. germination
1	No perforation (control)	Not sealed	19
2	Entire husk removed	Not sealed	72
3	Proximal region of lemma	Not sealed	53
4		5 seconds	44
5		72 hours	43
6	Median region of lemma	Not sealed	45
7		5 seconds	39
8		2 hours	44
9		72 hours	48
10	Distal region of lemma	Not sealed	25
11		5 seconds	11
12		72 hours	22

A second experiment (Table III) was designed in order to verify the results of the first experiment and to investigate more fully the consequences of sealing the perforations in the husk after various intervals from the time of excision. In general, this experiment confirmed the main conclusions drawn from the first: for a population of seeds, excision of a small part of the husk is not as effective as removing the entire husk, and a perforation is more efficient in breaking dormancy when the site is near to the embryo. In the second experiment a perforation in the distal position was not entirely ineffective as shown previously, but nevertheless it was much less effective than at the other sites. Little effect of sealing the perforations in the husk after different periods was demonstrated in this experiment except in the distal position and possibly to a smaller extent in the median position, where it was shown that the effect of sealing was only inhibitory if carried out within a few seconds of perforating the husk.

Attempts to detect the presence of a germination inhibitor in dormant seed

There is a growing body of evidence which shows that seed dormancy in many species is due to the presence of inhibitory substances in various parts of the dispersal unit (Evanari, 1949). In at least one case, i.e. in *Xanthium*, it was previously thought that dormancy could be explained entirely on the

basis of the impermeability of the seed coat to oxygen (Crocker and Barton, 1953, pp. 78, 122); but it has now been shown (Wareing and Foda, 1957) that inhibitors are also important in this species. Thus increasing attention is now being paid to all species, including cereals. The dormancy of oat seeds has been explained (Elliot and Leopold, 1953) by the presence of a water-soluble amylase and germination inhibitor in the husk. (Peers, 1958, has since found other water-soluble germination inhibitors in oat husk, but these were said to affect other species and not the oat seed itself.) Kommedhal *et al.* (1958) found evidence which suggested the presence of a water-soluble germination inhibitor in the husk of dormant wild oats; though in this case some doubt was raised as to whether the inhibitory material was produced by the husk itself or by micro-organisms associated with it. Black (1959) has since confirmed the presence of inhibitors in the husk of this species and has shown that probably the same inhibitors are also present in the seminal tissue. Furthermore, Black presented evidence which indicates that the main inhibitory effect of the husk in this species is due to its action as a barrier to the leaching of water-soluble inhibitors from the caryopsis itself. Nevertheless, the dormancy mechanism in wild oats cannot depend solely on this effect of the husk in combination with internal germination inhibitors since no difference of inhibitor content could be shown between dormant and after-ripened seed.

In the present study a number of attempts have been made to extract germination inhibitors from various parts of rice seed. Since no evidence of inhibitors has been found, these experiments will not be discussed in detail, but the design and results of some of them are presented briefly in Tables IV and V.

The possibility that the effect of the husk is due to its action in preventing the leaching of inhibitors from the caryopsis as suggested by Black (1959) for wild oats has also been investigated in the present studies on rice. The hypothesis on which these experiments is based is that dormant seeds should only be capable of germinating when the leaching of the inhibitors is facilitated.

In dehusked or perforated seeds leaching should occur when the dehusked or perforated seeds are set to germinate in contact with liquid water but not when they are in contact with humid air. Black found that very little germination occurred in dormant dehusked seed of wild oats in humid air although considerable germination was possible in water. In an experiment designed to test this possibility in rice, dehusked dormant and dehusked non-dormant seed was set to germinate in the apparatus shown in Fig. 2. Control treatments using intact and dehusked dormant and non-dormant seed were set up in the usual way in Petri-dishes, but incubated at 35° C. instead of 32° C. in order that these treatments should be comparable in temperature with those in humid air. It is evident from the results shown in Table VI that dormant dehusked rice seeds are capable of germination when not in contact with liquid water.

TABLE IV

Summary of inhibitor-extraction experiments

Quantity of seed extracted	Parts of seed extracted	Extraction technique	Germination test
225 g. dormant seed (i.e. about 10,000 seeds).	Two fractions treated separately: (husk + bran + embryos) (endosperm).	Shaken for 24 hrs. in 400 ml. distilled water at about 27° C. in the dark. Filtered. Filtrates made up to 400 ml. 200 ml. of filtrate autoclaved and thereafter treated separately from the remainder.	4 ml. of extract added to 6 ml. distilled water in each Petri-dish. Standard germination test carried out on non-dormant seed. No inhibition was shown by either the unsterilized or autoclaved extract.
Ditto	Ditto	Shaken for 24 hrs. in 200 ml. ethyl ether. Extract evaporated to dryness under vacuum at 40° C. Water-soluble residue taken up in 400 ml. distilled water. 200 ml. of this autoclaved and thereafter treated separately from the remainder.	Ditto.
460 g. dormant seed.	All fractions except husk discarded. Husk ground in hammer mill.	Shaken with 160 ml. distilled water for 2 hrs. in the dark. Filtered. Filtrate made up to 140 ml. with distilled water.	Germination tests carried out in sealed Ehrlenmeyer flasks. 2 ml. husk extract added to 3 ml. distilled water in each flask containing 25 seeds of a partially dormant population. Treatments split in 3 ways: aerobic and anaerobic conditions (N_2 or air blown through the flasks at 8-hour intervals); light and dark; intact and dehusked seeds. Results shown in Table V. Inhibition caused by darkness and anaerobic conditions but not by extract.
200 g. dormant seed. Control: 200 g. non-dormant seed.	Husk discarded. Pericarp, testa, endosperm, and embryo extracted together.	Soaked in 100 ml. distilled water for 48 hrs. at 3° ± 2° C. Filtered. Evaporated to dryness under vacuum for 20 hrs. at 23° ± 2° C. Made up to 10 ml.	Germination test carried out using 50 dehusked non-dormant seeds and 10 ml. extract in a Petri-dish in diffuse daylight. Neither the dormant nor non-dormant extract inhibited germination. Both extracts severely and equally inhibited subsequent growth (possibly due to growth of micro-organisms or the high osmotic concentration of the extract).

TABLE V

The effect of a water extract of the husk of dormant rice on the germination of newly harvested rice seed in nitrogen or air and in the light or in the dark

		Per cent. germination			
		Dark		Light	
		Air	Nitrogen	Air	Nitrogen
Rice seed with intact husk	Germinated in water	12	0	12	2
	Germinated in extract	4	0	10	0
Rice seed with husk removed	Germinated in water	50	24	60	38
	Germinated in extract	80	50	92	60

Black found that if part of the husk was removed in wild oat seeds considerably more germination was obtained if the exposed caryopsis was placed in contact with moist filter-paper than if the seed was orientated so that the dehusked portion faced upwards and away from the filter-paper. It was pointed out that these results are consistent with the hypothesis that the husk acts by preventing leaching. A similar type of experiment has now been

TABLE VI

A comparison of the germination of dormant and non-dormant seeds in humid air and in water

Treatment no.	Seed	Germination conditions	Per cent. germination
1	Dormant, dehusked	Moist filter-paper	72
2	Non-dormant, dehusked		95
3	Dormant, intact		6
4	Non-dormant, intact		97
5	Dormant, dehusked	Humid air	83
6	Non-dormant, dehusked		97

TABLE VII

The effect of the orientation of perforated dormant seed on germination

Treatment no.	Seed	Orientation of embryo (n.b. perforations are at the opposite end of the seed to the embryo)	Per cent. germination
1	Non-dormant, intact	down	98
2	Non-dormant, intact	up	99
3	Dormant, intact	down	6
4	Dormant, intact	up	10
5	Dormant, perforated	down	39
6	Dormant, perforated	up	23

carried out on rice. Seeds were set to germinate on 6 cm. 'sterimats' (Ford's FCB) in 9 cm. Petri-dishes irrigated with 20 ml. distilled water. This amount of water was not sufficient to reach the top of the sterimat and thus the seeds were in contact with the water held in the mat by capillarity. Twenty-five seeds were held in small depressions in the mat at 1 cm. intervals so that their long axes were in a vertical position. Otherwise replication and other conditions were the same as in a standard germination test. In some treatments the husk was perforated, the excision being made in the distal-lemma position, i.e. at the end of the seed away from the embryo.

The list of treatments together with the results are shown in Table VII. It will be seen that these results cannot easily be explained in terms of a leaching hypothesis. The orientation of the seed had little effect, but in fact

a slightly higher value was obtained when the perforation of the seed was uppermost (Treatment 5) than when the perforation was in contact with liquid water (Treatment 6).

Attempt to detect a germination stimulator in non-dormant seed

Recent discoveries have shown that not only are germination inhibitors involved in the control of seed dormancy in many species, but in some cases at least, substances stimulating germination are also implicated. Luckwill (1952) showed that growth-promoting substances are important in controlling the germination of apple seed. More recently Villiers and Wareing (1960)

TABLE VIII

The effect of water extracts of non-dormant rice seed on the germination of dormant rice seed

Treatment no.	Seed	Medium used in germination test	Per cent. germination
1	Intact	Distilled water	6
2	Intact	Husk extract	8
3	Intact	Bran extract	4
4	Intact	Endosperm extract	10
5	Dehusked	Distilled water	71
6	Dehusked	Husk extract	71
7	Dehusked	Bran extract	59
8	Dehusked	Endosperm extract	67

showed that the main factor controlling germination in *Fraxinus excelsior* is a germination stimulator which performs its function by overcoming the influence of a germination inhibitor which is also present. An experiment was therefore carried out to investigate the possibility of detecting the presence of a germination stimulator in rice seed which has completely broken dormancy.

460 g. of seed which had been stored at a mean temperature of approximately 18° C. for 4 months after harvest and had completely broken dormancy was milled in a Minghetti sampling mill. After milling, the husk and bran fractions were separated by sieving. Three fractions were then available: husk, bran, and endosperm; most of the embryos were included in the bran fraction. After the husk and the endosperm fractions had been ground in a hammer mill, all three fractions were extracted separately for 2 hours in 320 ml. water at 27° C. After filtration, 140 ml. was recovered from each fraction. Standard germination tests were carried out on a sample of the same seed except that in this case the seed had been stored since harvest at 3°±2° C. in order to maintain most of the seeds in a dormant condition. The extracts were added to the Petri-dishes at the rate of 4 ml. extract to 6 ml. distilled water.

Details of the treatments together with the results of the germination tests are listed in Table VIII. It will be seen that the extracts had very little effect on either intact or dehusked seed. A possible exception to this was a

slight inhibitory action of the bran extract. Thus this experiment did not provide any evidence to suggest the presence of a water-soluble germination-stimulating substance in non-dormant seeds.

DISCUSSION

The fact that it has not been found possible to extract substances which either inhibit or stimulate germination does not preclude the existence of such substances, and final judgement of this aspect of the problem will have to await further work. Nevertheless, the suggestion implicit in the results of the extraction experiments, that the main inhibitory action of the husk is not due to its possible content of inhibitors, is also supported by the surgical experiments. If inhibiting substances in the husk were important, it is unlikely that the removal of less than 2 sq. mm. of husk would have such a marked stimulatory effect. In contrast to rice, there is evidence that similar treatments of the covering structures of seeds in which inhibiting substances are known to be present do not stimulate germination: e.g. Luckwill (1952) showed that rupturing the testa and endosperm in apple seeds does not cause an increase in germination.

One possibility is that the husk prevents germination in newly harvested seed by acting as a barrier to the diffusion of some substances either to or from the embryo; this idea has much to support it from the results of the surgical experiments. The present work has excluded the possibility that entry of water is prevented by the husk of dormant seeds and it is therefore necessary to consider other substances. As no water-soluble or ether-soluble inhibiting substances have been detected in the endosperm, bran, and embryo fractions, it is suggested that it is not probable that the husk acts by restricting the outward diffusion of such a substance. The indirect evidence of the experiments concerned with leaching also supports this suggestion. Furthermore, although the interpretation of the results cannot be considered infallible since the extent of possible leakages through the seals is not known, the surgical treatments where the perforations were sealed with paraffin also support this idea: sealing had little effect on the stimulation caused by the initial excision—unless carried out within a few seconds at the distal site. The hypothesis of the outward diffusion of a water-soluble or ether-soluble inhibitor thus appears unlikely. Such a substance could still be involved, however, providing one makes a further postulate that it is very unstable—for instance, if it were easily oxidatively destroyed. But if one excludes the possibility of water or an inhibitor of the type just described as being the operative substance, the next most likely possibilities are that the husk restricts the outward diffusion of some volatile or gaseous inhibitor, or it restricts the inward diffusion of some gaseous substance necessary for some metabolic process leading to loss of dormancy; either of these hypotheses would make the inhibitory effect of sealing perforations after a few seconds more understandable. These possibilities will be considered in greater detail in the next contribution.

Although dormancy can be removed from most rice seed by dehusking, this is not an ideal method for use by plant breeders and others wishing to germinate seed immediately after harvest. It suffers from two main defects: first, considerable care has to be exercised in removing the husk from the grain if damage to the embryo is to be avoided; secondly, the dehusked grain is usually subject to fungal and bacterial attacks when set to germinate, whereas the intact seed is not. The manner in which the husk carries out this protective action is not clear. Attempts made with the collaboration of Dr. M. G. R. Hart of this station have failed to detect the presence of an antibiotic in the husk which is active against micro-organisms associated with germinating rice.

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Cell Division and Expansion in the Growth of the Shoot Apex

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WITH THREE FIGURES IN THE TEXT

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SUMMARY

The number and the total volume of cells produced in small portions of the apical region of the shoot have been measured at different stages of development.

In non-vernalized seedlings of winter rye, grown in July, the cells in the portions examined divided about once every 1·8 days during the first week of growth, and there was about a 2·2-fold increase in the volume of each cell generation. Four weeks later the apices were still vegetative, but the rate of division in the portions then examined had fallen to once every 5·8 days and there was slightly less than a 2·2-fold increase in the volume of each generation. The average cell volume decreased as the apices developed.

In vernalized seedlings more cells were produced in unit time during the first 4 days of growth than in non-vernalized seedlings, but, as in the latter, the rate of production fell during vegetative development. The rate of cell expansion in the vernalized seedlings was probably slightly lower than in the non-vernalized seedlings during the first 4 days of growth and tended to increase during vegetative development. At the time of transition to reproductive growth the cells were dividing about once every 2·0 days and there was about a 2·2-fold increase in the volume of each succeeding generation. After transition the rates increased. From 4 to 8 days after transition the cells divided once every 1·4 days and there was then a 2·4-fold increase in the volume of each succeeding generation. Subsequently, the rate of division changed little but the rate of expansion decreased.

Higher rates of division and expansion were found in the apices of lupin seedlings grown in July than in November. In the portions of the meristem examined during the first 5 days of growth the cells divided about once every 1·3 days in July, but only once every 1·8 days in November. The increase in volume of each generation in the same time was slightly higher in July than in November. Both the rates of division and expansion decreased until transition to reproductive growth occurred, and then increased. About 5 days after transition the cells divided once every 1·4 days in July and once every 2·0 days in November. In both experiments there was a 2·4-fold increase in the volume of each generation. Both the rates of division and expansion decreased subsequently.

The data are discussed in relation to the changes in size which accompany the development of the shoot apex.

INTRODUCTION

In higher plants the vegetative development of the shoot apex begins during the maturation of the seeds. It is resumed as soon as the seed germinates and the production of leaf primordia at the apex continues thereafter in a regular sequence until the change to reproductive growth occurs. If the entire apex

becomes reproductive, leaf production ceases altogether, and a sequence of flower production usually follows.

Flower primordia are produced at a higher rate than leaf primordia in chrysanthemum (Schwabe, 1959). The rate of leaf production in this species is relatively unaffected by the prevailing growth temperature and it is accelerated by vernalization. In cucumber, on the other hand, the rate of leaf production is accelerated by the prevailing growth temperature and also by light (Milthorpe, 1959).

The present paper is concerned with the rates at which the cells multiply and expand in the apex of the shoot. The data illustrate the differences between these rates both in the vegetative and the reproductive phases of development, and the changes which occur in these rates as the apex develops. The effects of vernalization and of seasonal changes have also been studied.

MATERIALS AND METHODS

The experiments were performed with two species in which the entire apex is involved in the change from vegetative to reproductive growth. Winter rye, *Secale cereale* var. 'Petkus', was used in one series of experiments. Apices of both vernalized and non-vernalized seedlings were examined. The apices of the non-vernalized seedlings remained vegetative throughout the experiments, while those of the vernalized seedlings became reproductive about 4 days after the seedlings were sown. The annual lupin, *Lupinus albus*, was used in another series of experiments. In this species the apices became reproductive about 2 to 3 weeks after the seeds were sown.

All the seedlings were grown in boxes in John Innes compost in a glasshouse under natural light conditions and at a temperature of $20° \pm 3°$ C. The experiments with lupin were performed in November 1959 and in July 1960; the experiments with rye were both performed in July of these years. The untreated grain and the vernalized seedlings of rye were sown simultaneously, the latter having been kept previously for 12 weeks at 2° C.

The measurements were begun at the time of sowing, or very soon afterwards, and were continued at fixed intervals. The interval between the first and the second measurements is referred to as period 1; the interval between the second and the third as period 2 and so on. At the beginning of each period 20 seedlings were selected at random and each was dissected to reveal the apical region. The same total number of leaves and primordia was dissected away from each of the seedlings. The portion of the meristem which remained was excised by a cut made along the base of the largest remaining primordium. The total volume of 10 of the resulting fragments and the number of cells in the other 10 were then measured. This procedure was repeated at the end of the period on another 20 seedlings from which the same total of leaves and primordia was removed as at the beginning of the period. The increments observed in the number of cells and in their total volume indicated the average rates of multiplication and expansion during that period.

In each successive period a progressively larger number of leaves and primordia was dissected away. The total number removed had to be limited to that carried by the smallest of the seedlings selected. Since the seedlings grew at different rates, some much faster than others, the initial size of the fragments tended to increase from one period to the next.

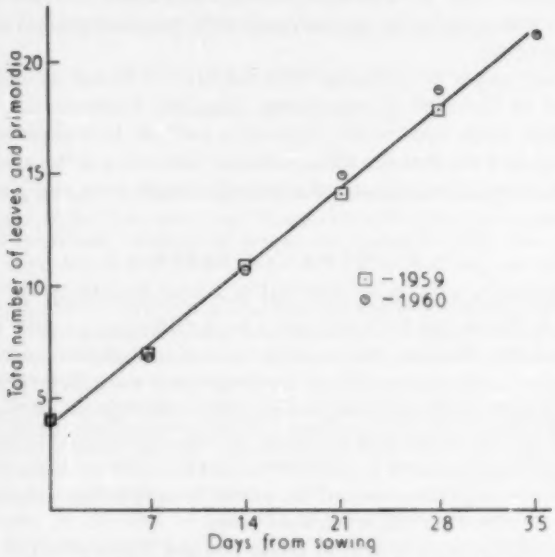


FIG. 1. Total number of leaves and primordia plotted against seedling age. Non-vernalized rye.

The dissection of the seedlings, the excision of the fragments, the determination of total volume and of cell number were all carried out in the manner described by Sunderland and Brown (1956). The values reported below are the means obtained from the ten measurements made on each occasion.

RESULTS

1. *Vegetative growth*

Non-vernalized rye. The rate at which leaf primordia were produced by the seedlings of non-vernalized rye is indicated by the data of Fig. 1. The rate fluctuated slightly as the seedlings developed, probably because they were not grown in constant conditions. Primordia appeared at an average rate of about one every 2 days. The rate was similar in both 1959 and 1960. During each period in which measurements were made, therefore, from 3 to 4 new primordia and embryonic internodes were produced.

In contrast to the constant rate of leaf production, the rates at which cells were produced and at which they expanded changed markedly as the apices

developed. This is shown by the data of Tables I and II. In the 1960 experiment, for example, about 122,000 cells were produced in period 1 in contrast to only 2,400 in period 5 (Table I). The average cell volume in the same experiment increased about 3·5 times in period 1 but did not change in period 5 (Table II). From these data it can be calculated that the cells produced in period 5 required about 10 days in which to double their size, whereas in period 1 each generation of cells expanded about 2·5 times in a little over one day. The length and breadth of the fragments examined also increased to a greater extent in the earlier than in the later periods (Table II).

TABLE I

Data for non-vernalized rye. Values at beginning of a period—A, values at end of a period—B

Period	Days from sowing	Total of leaves and primordia removed	Number of primordia on each fragment		Number of cells per fragment		B/A	Number of cell generations	Duration of one generation (days)
			A	B	A	B			
1959									
1	0	3	1·0	4·0	750	12,250	16·3	4·0	1·8
2	7	5	2·0	5·9	1,690	20,140	11·9	3·5	2·0
3	14	7	3·9	7·1	4,330	20,180	4·7	2·2	3·2
4	21	9	5·1	8·8	5,470	13,460	2·5	1·2	5·8
1960									
1	0	2	2·0	4·8	2,140	124,060	58·0	5·8	1·2
2	7	5	1·8	5·7	1,820	20,130	11·1	3·4	2·1
3	14	8	2·7	6·9	3,030	15,510	5·1	2·3	3·0
4	21	12	2·9	6·7	3,600	7,450	2·1	1·1	6·4
5	28	16	2·7	5·2	3,540	5,920	1·7	0·7	10·0

TABLE II

Data for non-vernalized rye

Period	Total volume per fragment $\times 10^{-3}$ mm.^{3}			Average cell volume $\times 10^{-3}$ mm.^{3}			Volume increase of each cell generation	Length μ			Diameter at base of fragment μ		
	A	B	B/A	A	B	B/A		A	B	B/A	A	B	B/A
1959													
1	1·12	25·7	22·9	14·9	20·9	1·40	2·18	102	407	4·0	122	341	2·8
2	2·13	32·0	14·9	12·7	15·9	1·25	2·13	192	520	2·7	—	—	—
3	5·13	29·5	5·7	11·8	14·6	1·24	2·19	324	570	1·8	—	—	—
4	7·01	19·3	2·8	12·8	14·4	1·12	2·15	371	565	1·5	—	—	—
1960													
1	3·32	665·0	200·0	15·5	53·6	3·45	2·47	167	4,770	28·6	216	680	3·1
2	2·59	34·2	13·2	14·2	17·0	1·19	2·10	205	508	2·5	174	390	2·2
3	4·07	22·4	5·5	13·4	14·4	1·08	2·06	265	593	2·2	187	288	1·5
4	4·80	10·1	2·1	13·3	13·6	1·01	2·00	312	473	1·5	182	209	1·1
5	4·35	7·3	1·7	12·3	12·4	1·00	2·00	288	407	1·4	175	195	1·1

The decreasing absolute and relative rates of growth which these measurements indicate are associated with the decreasing primordial size which accompanies the development of the apex. But there is clearly a marked

decrease in the average rates of cell multiplication and of cell expansion; the rates are higher in the primordia produced early than in those produced later. In this connexion the data obtained in period 1 of the two experiments are particularly relevant. It will be seen that the absolute and the relative rates of

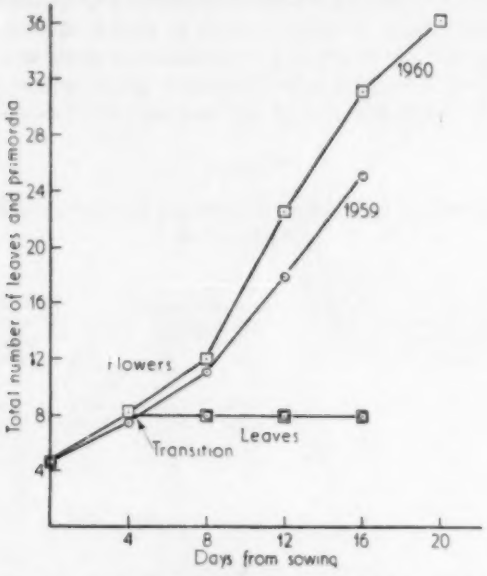


FIG. 2. Total number of leaves and primordia plotted against seedling age. Vernalized rye.

growth in period 1 were lower in 1959 than in 1960. This is because the fragments examined in 1960 carried an additional primordium, the third above the cotyledons. It is evident that the large difference in the growth-rates of the cells on the two occasions was due wholly to the growth of the third primordium since, as observed above, the growth-rates of the seedlings in the two experiments were very similar. The linear measurements given in Table II show that the third primordium increased about 4,600 μ in length and 460 μ in breadth in 7 days in contrast to the fourth primordium which increased, in the same time, only about 300 μ in length and 220 μ in breadth. This comparison shows that the rates of cell multiplication and expansion in leaf 3 are greater than in leaf 4 during the first 7 days after their formation, and the data are consistent with the view that the rates continue to decrease progressively in each primordium subsequently produced.

The rate of expansion decreases more rapidly than the rate of multiplication, and the average size of the cells in the meristem changes accordingly. It will be seen that the average cell volume of the fragments initially fell from 15 to 13×10^{-7} mm.^{3} in the four periods of the 1959 experiment and from 16 to

12×10^{-7} mm.^{3} in the five periods of the 1960 experiment (Table II). Had it been possible to make the measurements on fragments initially of the same size it is probable that the differences would have been more marked.

TABLE III

Data for vernalized rye. Transition occurred in period 2

Period	Days from sowing	Total of leaves and primordia removed	Number of primordia on each fragment A	Number of primordia on each fragment B	Number of cells per fragment A	Number of cells per fragment B	B/A	Number of cell generations	Duration of one generation (days)
1959									
1	0	3	1·5	4·5	1,400	14,270	10·2	3·3	1·2
2	4	5	2·5	6·1	3,110	13,700	4·4	2·1	1·9
3	8	7	4·1	10·9	4,720	38,210	8·1	3·0	1·3
4	12	9	8·9	16·3	13,960	101,510	7·3	2·8	1·4
1960									
1	0	3	1·7	5·2	1,460	15,810	10·9	3·4	1·2
2	4	7	1·2	5·0	1,430	5,570	3·9	1·9	2·1
3	8	10	2·0	12·6	2,620	19,850	7·6	2·9	1·4
4	12	19	3·6	12·2	7,400	63,830	8·6	3·1	1·3
5	16	28	3·2	8·3	6,810	53,520	7·9	2·9	1·4

TABLE IV

Data for vernalized rye

Period	Total volume per fragment $\times 10^{-9}$ mm.^{3} A	Total volume per fragment $\times 10^{-9}$ mm.^{3} B	B/A	Average cell volume $\times 10^{-7}$ mm.^{3} A	Average cell volume $\times 10^{-7}$ mm.^{3} B	B/A	Volume increase of each cell generation	Length μ A	Length μ B	B/A	Diameter at base of fragment μ A	Diameter at base of fragment μ B	B/A
1959													
1	2·81	30·8	10·9	20·0	21·6	1·07	2·03	169	501	3·0	—	—	—
2	3·79	19·7	5·2	12·2	14·4	1·18	2·17	245	497	2·0	—	—	—
3	4·83	51·9	10·7	10·2	13·6	1·32	2·21	323	820	2·5	—	—	—
4	21·45	185·3	8·6	15·4	18·3	1·18	2·13	583	1,248	2·1	—	—	—
1960													
1	2·68	37·3	13·9	18·4	23·6	1·28	2·14	168	602	3·6	179	340	1·9
2	1·77	8·1	4·6	12·4	14·6	1·18	2·18	192	409	2·1	155	204	1·3
3	2·70	31·4	11·6	10·3	15·8	1·53	2·31	246	778	3·2	164	267	1·6
4	10·11	148·7	14·7	13·7	23·3	1·71	2·37	388	1,079	2·8	223	560	2·5
5	12·40	131·2	10·6	18·2	33·5	1·34	2·24	367	1,016	2·8	246	566	2·3

Vernalized rye. During vernalization the number of leaf primordia on the embryonic shoot of the rye seedlings increased from 4 to an average of 4·6 (Tables I and III). About 700 cells were produced in the portion of the meristem above the third primordium, and their average volume was greater than that of the cells in the corresponding portion of the non-vernalized seedlings (Tables II and IV). As in the latter, however, primordia were produced at a constant rate after sowing (Fig. 2) and the relative increases in cell number and volume both fell from the first to the second period (Tables III and IV). It will be seen that the apices of the vernalized seedlings grew more rapidly than those of the non-vernalized seedlings. Primordia were produced at about

double the rate and cells were produced at a rate about 0·6 day higher than that in the non-vernalized seedlings (Period 1 1959, Table I; Period 1 1959, 1960, Table III).

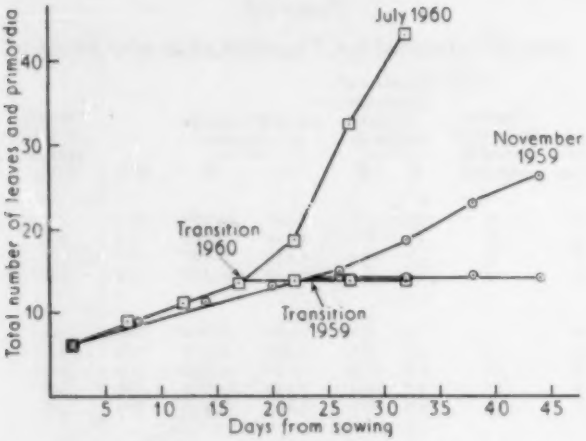


FIG. 3. Total number of leaves and primordia plotted against seedling age. Lupin.

The rate of expansion was probably less affected than the rate of cell multiplication. In period 1 of the two experiments with vernalized seedlings the rate of expansion was slightly lower than in the corresponding period with non-vernalized seedlings, and it tended to increase slightly from the first to the second period. The average cell volume decreased markedly from the first to the second period.

Lupin. Seasonal changes affected the growth-rate of the apices of the lupin seedlings in the vegetative condition. Primordia were produced at a rate of one every 2 days in July and one every 3 days in November, but in both experiments the rate was constant (Fig. 3). The same number of primordia was produced in the two experiments so that the vegetative phase of development was shorter in July than in November.

The rates of cell multiplication and expansion were also different in the two experiments. In period 1 cells were produced at an average rate of one every 1·3 days in July but at a rate of one every 1·8 days in November (Table V). In both experiments the rate decreased from the first to the fourth period, when vegetative growth ceased, and it decreased more rapidly in November than in July.

The data of Table VI show that the average cell volume increased about 46 per cent. in period 1 in July, but only 28 per cent. in period 1 in November. The rate of expansion decreased from the first to the fourth period in both experiments, and it decreased more slowly in November than in July. There

was a marked decrease in the average cell volume from the first to the fourth period in July and probably a slight decrease in November.

TABLE V

Data for lupin. Transition occurred in period 4

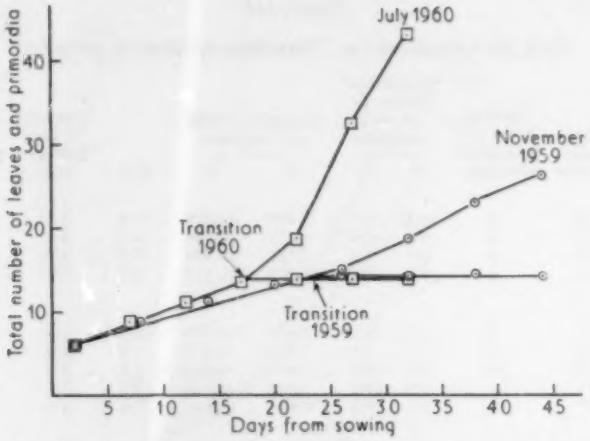
Period	Days from sowing	Total of leaves and primordia removed	Number of primordia on each fragment		Number of cells per fragment		B/A	Number of cell generations	Duration of one generation (days)
			A	B	A	B			
1959									
1	2	5	1·1	3·7	2,400	25,480	10·6	3·3	1·8
2	8	7	1·7	4·2	4,600	36,880	8·0	3·0	2·0
3	14	10	1·2	2·2	2,510	14,700	5·9	2·5	2·4
4	20	12	1·2	3·1	2,640	10,930	4·1	2·0	3·0
5	26	14	1·1	5·3	2,870	21,620	7·5	2·9	2·0
6	32	18	1·3	5·2	7,400	21,670	2·9	1·5	4·0
7	38	22	1·2	4·2	6,120	16,120	2·6	1·3	4·6
1960									
1	2	5	1·2	3·7	1,400	22,010	15·8	4·0	1·3
2	7	7	1·7	4·1	3,010	27,770	9·2	3·2	1·6
3	12	10	1·1	3·5	3,240	19,550	6·0	2·5	2·0
4	17	12	1·5	7·4	5,290	33,900	6·4	2·6	1·9
5	22	16	3·4	16·5	8,040	104,510	13·0	3·6	1·4
6	27	25	7·5	18·2	32,990	165,330	5·0	2·2	2·3

2. *Reproductive growth*

Vernalized rye. Flower primordia were produced at a higher rate than leaf primordia. In 1959 they were produced at an average rate of one in about 17 hours and, in 1960, one in about 14·5 hours (Fig. 2). The rate of production of flower primordia increased slowly for a short time after transition from vegetative to reproductive growth had occurred, and then rose sharply. This transition occurred in period 2 of the experiments with rye (Tables III and IV). The rapid increase in the rate of flower production occurred in the following period. The data show that the rapid increase in the rate of flower production was accompanied by large increases in the rates of cell multiplication and expansion. In the 1960 experiment, for example, the relative increase in the number of cells in period 3 was double that in period 2, a difference which involved more than 13,000 cells (Table III). The average cell volume in the same experiment increased about 53 per cent. in period 3 but only 18 per cent. in period 2, and there were corresponding changes in the linear dimensions of the fragments examined in these periods. It will be seen that the rate of cell multiplication increased until it was almost as high as in the early stages of vegetative growth; the increase in the rate of expansion was even greater than that observed in the early stages of vegetative growth.

The behaviour of the meristem in the later stages of reproductive growth differed from its behaviour in the later stages of vegetative growth. About the same number of cell generations were produced in periods 4 and 5 as in period 3. There was, however, a change in the rate of expansion. In the 1959

double the rate and cells were produced at a rate about 0·6 day higher than that in the non-vernalized seedlings (Period 1 1959, Table I; Period 1 1959, 1960, Table III).



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The rates of cell multiplication and expansion were also different in the two experiments. In period 1 cells were produced at an average rate of one every 1·3 days in July but at a rate of one every 1·8 days in November (Table V). In both experiments the rate decreased from the first to the fourth period, when vegetative growth ceased, and it decreased more rapidly in November than in July.

The data of Table VI show that the average cell volume increased about 46 per cent. in period 1 in July, but only 28 per cent. in period 1 in November. The rate of expansion decreased from the first to the fourth period in both experiments, and it decreased more slowly in November than in July. There

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Data for lupin. Transition occurred in period 4

Period	Days from sowing	Total of leaves and primordia removed	Number of primordia on each fragment		Number of cells per fragment		B/A	Number of cell generations	Duration of one generation (days)
			A	B	A	B			
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1	2	5	1·1	3·7	2,400	25,480	10·6	3·3	1·8
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4	20	12	1·2	3·1	2,640	10,930	4·1	2·0	3·0
5	26	14	1·1	5·3	2,870	21,620	7·5	2·9	2·0
6	32	18	1·3	5·2	7,400	21,670	2·9	1·5	4·0
7	38	22	1·2	4·2	6,120	16,120	2·6	1·3	4·6
1960									
1	2	5	1·2	3·7	1,400	22,010	15·8	4·0	1·3
2	7	7	1·7	4·1	3,010	27,770	9·2	3·2	1·6
3	12	10	1·1	3·5	3,240	19,550	6·0	2·5	2·0
4	17	12	1·5	7·4	5,290	33,900	6·4	2·6	1·9
5	22	16	3·4	16·5	8,040	104,510	13·0	3·6	1·4
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Vernalized rye. Flower primordia were produced at a higher rate than leaf primordia. In 1959 they were produced at an average rate of one in about 17 hours and, in 1960, one in about 14·5 hours (Fig. 2). The rate of production of flower primordia increased slowly for a short time after transition from vegetative to reproductive growth had occurred, and then rose sharply. This transition occurred in period 2 of the experiments with rye (Tables III and IV). The rapid increase in the rate of flower production occurred in the following period. The data show that the rapid increase in the rate of flower production was accompanied by large increases in the rates of cell multiplication and expansion. In the 1960 experiment, for example, the relative increase in the number of cells in period 3 was double that in period 2, a difference which involved more than 13,000 cells (Table III). The average cell volume in the same experiment increased about 53 per cent. in period 3 but only 18 per cent. in period 2, and there were corresponding changes in the linear dimensions of the fragments examined in these periods. It will be seen that the rate of cell multiplication increased until it was almost as high as in the early stages of vegetative growth; the increase in the rate of expansion was even greater than that observed in the early stages of vegetative growth.

The behaviour of the meristem in the later stages of reproductive growth differed from its behaviour in the later stages of vegetative growth. About the same number of cell generations were produced in periods 4 and 5 as in period 3. There was, however, a change in the rate of expansion. In the 1959

experiment the increase in the average cell volume fell from 32 per cent. in period 3 to 18 per cent. in period 4, and in the 1960 experiment from 71 per cent. in period 4 to 34 per cent. in period 5.

Lupin. The growth of the apices of the lupin seedlings in the reproductive phase was similar in many respects to that observed in rye. Flower primordia were produced at a higher rate than leaf primordia and the rate of production was not constant. Seasonal changes affected the growth of the apices in the reproductive phase as in the vegetative phase. In July primordia appeared at an average rate of about one every 12 hours, but in November the rate was about one every 32 hours (Fig. 3).

TABLE VI

Data for lupin

Period	Total volume per fragment $\times 10^{-2}$ mm.^{3}			Average cell volume $\times 10^{-2}$ mm.^{3}			Volume increase of each cell generation	Length μ			Diameter at base of fragment μ		
	A	B	B/A	A	B	B/A		A	B	B/A	A	B	B/A
1959													
1	2·39	32·6	13·6	9·9	12·8	1·28	2·16	111	203	1·8	262	518	2·0
2	3·67	40·3	11·0	8·0	10·9	1·37	2·22	138	230	1·7	327	500	1·5
3	1·95	13·9	7·1	7·8	9·4	1·20	2·15	121	199	1·6	253	405	1·6
4	2·39	10·0	4·2	9·0	9·2	1·02	2·07	115	191	1·7	246	343	1·4
5	2·34	19·0	8·1	8·2	8·8	1·08	2·03	122	302	2·3	225	392	1·7
6	5·54	33·2	4·2	7·5	10·7	1·45	2·45	167	340	2·0	274	393	1·4
7	4·13	13·4	3·3	6·7	8·3	1·27	2·31	145	283	2·0	208	320	1·5
1960													
1	1·76	40·5	23·0	12·6	18·4	1·46	2·20	88	184	2·1	263	502	1·9
2	3·59	38·1	10·6	11·9	13·7	1·15	2·09	104	216	2·1	303	513	1·7
3	2·91	19·4	6·7	9·0	9·9	1·12	2·07	99	224	2·3	258	437	1·7
4	3·04	33·5	11·0	5·8	9·9	1·72	2·44	120	323	2·7	264	481	1·8
5	6·21	176·0	28·4	7·7	16·9	2·18	2·47	160	796	5·0	288	805	2·8
6	34·90	218·6	6·3	10·6	13·2	1·26	2·21	384	838	2·2	437	880	2·0

Transition from vegetative to reproductive growth occurred in period 4 of the experiments with lupin (Tables V and VI). As in rye, the rates of cell multiplication and expansion both increased in the period following transition. These rates were higher in July than in November. The rate of multiplication eventually attained in both experiments was almost as high as in the early stages of vegetative growth; the rate of expansion actually exceeded that observed during early vegetative growth.

The behaviour of the lupin apices in the final period differed from that of the rye apices. In both experiments with lupin the rate of cell multiplication decreased rapidly after the peak had been reached although there was little change in the rate of production of primordia. The rate of multiplication observed towards the end of reproductive growth was lower than that observed at the end of vegetative growth. The rate of expansion also decreased in both experiments and was almost as low finally as at the end of vegetative growth.

DISCUSSION

The apex of the shoot exhibits a characteristic cyclic pattern of leaf production. In rye and lupin the apex is dome-shaped. In each plastochron the dome increases in size by cell division and expansion and from the enlarged mass a new primordium is split off laterally, and a new portion of the axis basally. The residual cells constitute the new dome for the next plastochron.

Previous reports have shown that the rates of cell division and of cell expansion are low in the dome. Sunderland and Brown (1956) found that division occurred about once every 3 days in the lupin. This rate was obtained from measurements made on apices which were completing vegetative growth. The present data show that the rates of division and expansion are relatively low at this stage. The rates are continually changing, however, as the apex develops and they are relatively high immediately after germination and after the onset of reproductive growth.

The rates reported here are, of course, only average rates which include the cells in the dome and in several primordia and embryonic internodes. It is highly probable, however, that these average rates approximate to those in the dome, and that the changes observed during development are indicative of corresponding changes in the dome. The average values reported here show that there is a progressive decline in the rates of multiplication and expansion in each successive primordium generated by the dome. It may be concluded therefore that these rates in the dome also decline as it develops. The rate of expansion declines relatively more rapidly than the rate of multiplication, indicating that the average cell size in the dome also decreases as it develops. Preliminary observations made directly on the dome and its products confirm these conclusions. A decrease in cell size during the development of the apical meristem of tomato has also been found by Whaley, who cites other examples (Whaley, 1939), and a progressive change in the relative growth-rate of successive primordia, estimated from changes in dry weight, has also been reported by Williams (1960).

Although the average rate of division and the average cell size both decrease during the vegetative development of the dome, the latter increases in size. Some of the linear measurements given in Table II reflect the change. It will be seen that the length of the fragments was 167 μ, and the diameter 216 μ, in period 1 of the 1960 experiment. Each of the fragments carried an average of two leaf primordia, the larger of which completely enclosed the dome and the other primordium. The dimensions given therefore include the larger primordium, and it is evident that the length from the tip of the dome to the base was actually less than 167 μ. Seven days later the fragments examined were each 205 μ long and 174 μ in diameter. Each fragment, although then much longer than in period 1, carried slightly less primordial tissue, and the larger primordium did not enclose the dome.

The elevation of the dome is frequently ascribed to an increase in the expansion of the central cells in the longitudinal direction (Vaughan, 1955).

In lupin and rye there is also an increase in the number of cells in the dome as it develops. The average number of cells in the dome in lupin increases from about 500 to 1,500 in the course of vegetative development and an even greater increase occurs in non-vernalized rye. On the other hand, the size of each primordium, and the number of cells in it, progressively decrease as the dome develops. The size of the dome therefore increases because at the differentiation of each primordium a progressively smaller proportion of the cells in the dome is given over to the new primordium and a correspondingly larger proportion remains to constitute the new dome.

With the onset of reproduction the size of the dome increases rapidly and its shape may change. In rye the elongated spike characteristic of grass apices begins to form. In lupin the dome increases in size but there is little change in shape. An examination of the linear dimensions of the fragments at the beginning of periods 2 and 3 (Table IV) and periods 4 and 5 (Table VI) will indicate the extent of the change.

The cyclic behaviour of the dome is lost in the reproductive phase. There is no reconstitution of the dome after the differentiation of each flower primordium. Instead there is a rapid formation of primordia along the flanks of the much enlarged dome. The size of the primordia remains small relative to that of the dome. The data presented here indicate that the rapid formation of primordia is accompanied by large increases in the rates at which the cells multiply and expand. These increases suggest a corresponding increase within the reproductive dome. Thus, at the time of transition it is highly probable that a large number of cells in the dome which have been dividing slowly start to divide rapidly. The rate of expansion also increases and to a greater extent than division, suggesting that the cells in the reproductive apex are more vacuolated than those in the vegetative apex.

After the initial burst of reproductive growth it is highly probable that the rates of division and expansion again decrease in the dome. This is certainly so in lupin. It is possible, however, that the rate of division in the reproductive dome of rye remains high until most of the primordia have been produced. The interpretation of the results obtained in the final periods of measurement with both species, however, is complicated by the comparatively large size of the fragments examined.

The rates of division and expansion in the dome are affected by seasonal changes and by vernalization. The data show that, in lupin, the development of the apex is similar in July and in November; it is merely more rapid in summer conditions as a result of increased rates of division and expansion. Vernalization also has the effect of increasing the rate of division in the dome and hence of increasing the rate of leaf production.

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An Automatic Device for Measuring Leaf Area

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WITH THREE FIGURES IN THE TEXT

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SUMMARY

The design and construction of a digital device, which measures the area of leaves by scanning with a rapidly moving spot of light, is described.

INTRODUCTION

AUTOMATIC planimeters described in the literature have, with one exception (Winter *et al.*, 1956), been analogue devices measuring some continuously variable quantity (light interception, air flow) related to area. This system is simple in principle but elaborate precautions are required to eliminate errors arising from variations in mains voltage, non-linearity and fatigue of photocells, stray light, contamination by mud from the leaves, non-uniformity of the light beam, and variations in leaf transmission. Some of these sources of errors are avoided by using air instead of light. Unfortunately, the airflow planimeter (Jenkins, 1959) is unsuitable for estimating large areas because of the great air flow required and because the null adjustment limits the speed of operation.

In crop studies the total area of a number of leaves in a sample is usually of more interest than the individual values. Unfortunately, it is impossible to make an analogue device give the total of a series of determinations unless the information is first converted into digital form. A digital instrument has greater potentialities; such a device would count the number of elements (unit areas) in the leaf, or series of leaves, and register the result. As the relation between counts and leaf area depends only on the size of the element, which is fixed by the dimensions of the instrument and the accuracy of the counting, it is independent of the sources of error mentioned above. These advantages are offset by the increased complexity of the system and it was decided to construct a digital planimeter suitable for sugar-beet as a test of the method.

Manual methods based on this principle were described by Winter *et al.* (1956).

DESIGN

To count the number of unit areas covered by a leaf, each area must be examined in sequence by a scanning system. Similar problems are encountered in television practice. The modern solution, the electronic camera tube, is unsuitable for our purpose; it is expensive, requires complex ancillary

apparatus, and is unnecessarily rapid. Baird's original method, the scanning disk, is cumbersome and gives low definition. The 'flying spot' method, in which the field is scanned by a beam of light reflected from mirrors mounted on a rotating drum, is simple, and fast enough.

The beam of light passes at a constant rate across the field, sweeping out a series of 'lines'. It also moves slowly at right angles to the line. Successive tracks are thus separated by a constant distance, and each line corresponds to an area defined by its length and the distance between corresponding lines.

The mirror drums used in the television system give a linear scan only when the angle swept by the beam is small. The line-scan drum was therefore replaced by a plane mirror rocked by a suitably shaped cam. This gives a linear scan over an angle of 50°. The repeated scanning of the field used in television was superfluous and the rotating field-scan drum was replaced by a rack and pinion which translates the entire scanner across the field. A moving belt carrying the leaves past a fixed scanner might have been more rapid but was considered to be too complicated for the prototype.

The light beam is interrupted by a toothed wheel mechanically linked to the scanner, so dividing the lines into a series of equal segments, each with an illuminated and a dark moiety. Each segment, and therefore each pulse of light, corresponds to a unit area. The use of an interrupted light beam also simplifies the amplifier and makes the instrument unresponsive to stray light. In the television raster, the return stroke is suppressed, the lines are all in the same direction, and the elements do not overlap. With the rocking mirror this is not possible; the forward and return strokes are identical and the raster is overlaid by its mirror image. Each pulse therefore corresponds to the sweeping out of half the unit area. A raster formed on the screen of a cathode-ray tube has been used as a source of a flying spot, particularly in microscopy, but the high voltages required to give an adequate light intensity and the complex ancillary equipment make this method unsuitable.

The mechanical system used is reversible in that it will project either an image of the field on a photocell, the entire field being illuminated, or an image of a light source on the field, a photocell being arranged to receive the light reflected. Both arrangements have some advantages, but as it is easier to observe the behaviour of the scanner with the latter arrangement it was adopted, although, in consequence, it was necessary to shade the field from strong light to prevent swamping of the photomultiplier.

The electrical pulses from the photomultiplier constitute analogue information concerning the amount of the light pulse falling on the leaf, and this must be sorted into two classes, 'on' and 'off', by a discriminator circuit, and the number in the 'on' class counted. Any error in the sorting will give an error in counting. The fully 'on' pulses are an order of magnitude smaller than the fully 'off' pulses (see Table I) and can easily be selectively rejected by a simple discriminator. The number of 'on' pulses is then obtained by subtracting, electronically, the discriminator output from the total number of pulses generated. With a spot of finite size, the change between the extreme

TABLE I

Reflectivity of various surfaces

Surface	Signal at V1 anode
(Noise)	(< 2 V)
Black paper	3 V
Green leaf	3–5 V
Dead leaf	10 V
Yellow leaf	10 V
White paper	40 V
Ballotini	60 V

pulse heights corresponds to a significant range of spot position. Any change in sensitivity or discriminator setting will be reflected in a change in the proportion of the spot which must fall on the leaf for the element to be counted. There is therefore a possible systematic error whose direction and magnitude vary with discriminator setting. Mathematical analysis of the situation is difficult but the percentage error decreases as the perimeter per unit area and the relative size of the element decreases. The first depends on the species of plant being investigated, e.g. measuring narrow, deeply lobed, or dissected leaves is difficult, and the second is restricted by the need to scan an adequate area per unit time at a pulse repetition frequency within the range of the scaler and other circuits.

If the scanning spot were a mathematical point, an infinitely small movement of the spot would change the pulse height from the fully 'off' to the fully 'on' level and the sorting would be independent of discriminator setting over a wide range. This was not even approximately possible with the optical system available, as a spot several mm. square was needed to give enough light to operate the photocell. Some of the disadvantages of the analogue planimeter specified in the introduction are therefore present in the instrument, but their effects are secondary in nature and are easily made negligible in practice.

CONSTRUCTION

The planimeter is housed in a 'Dexion' frame covered with hardboard. Two parallel lengths of 'Dexion 112' running horizontally along the length of the box act as running rails for the scanning carriage. A slot at the bottom of the case admits the trays in which the leaves are placed. The amplifier and power supplies are built on standard 19-in. chassis mounted on the top of the instrument case.

The scanning carriage (Fig. 1) is a light metal frame running on two pairs of wheels and bearing a lamp house at one end, a gear box and cam assembly at the other, and a rocking mirror and driving motor in the centre. The carriage is connected to the amplifier and power supplies by flexible cables. The lamp house contains two adjustable lamp-condenser units and a toothed interrupter disk. This disk has an outer set of twelve wide slots and an inner set of twelve narrow slots. The former have a 1:1 and the latter a 9:1 tooth-slot ratio. The beam of light from the smaller lamp passes through the narrow slots and falls directly on a photocell mounted opposite a window in the lamp house wall. This gives a train

of narrow electrical pulses corresponding to the total number of light pulses. Light from the larger lamp passes through a slit and a 5-cm. anastigmat lens to emerge as a slightly convergent beam parallel to the direction of motion. The beam is turned through 90° by a fixed mirror to fall on a mirror mounted on an axle. This

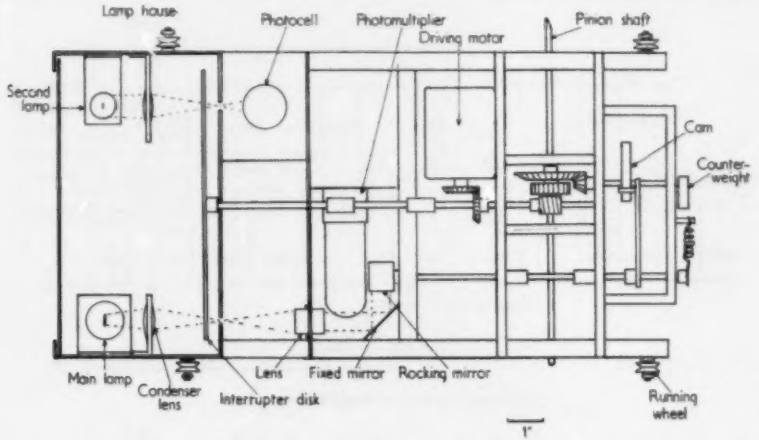

FIG. 1. Plan of scanning carriage with the top of the lamp house removed.

mirror turns the beam downwards on the leaf tray. The lens is adjusted to give a defined spot on the tray and the axle is oscillated through some 25° six times per second by an arm bearing a pin resting on a cam, sweeping the spot across the leaf. The cam is so shaped that the spot moves at a constant velocity proportional to the angular velocity of the cam shaft.

The leaf trays are painted white and covered with Ballotini glass beads, which have the property of reflecting a light beam back towards its source (here the mirror) whatever the angle of incidence on the reflecting surface. A photomultiplier mounted near the mirror converts the reflected light into electrical impulses. The leaves are held flat by a 'Perspex' plate and the plane of the scanning beam is offset from the normal so that light directly reflected from the 'Perspex' is directed away from the photomultiplier.

A rack engages either the top or the bottom of the driving pinion according to the direction of motion required. The pinion rotates at 120 r.p.m. giving a speed of 6 cm./sec. The field, 30 cm. × 50 cm., is scanned in approximately 9 secs. The cam is driven from the pinion shaft through a 3:1 step-up bevel gear, so one revolution of the cam (one scan in each direction) corresponds to a 1-cm. movement of the carriage. The interrupter disk rotates at 2,300 r.p.m. (approx.) giving 460 pulses per sec. and drives the pinion through a 19:1 worm gear.

The operation of the planimeter is controlled by a set of Post Office relays. Momentary closure of the start switch energizes a geared motor which rotates a crank until the rack engages the opposite side of the pinion, so reversing the motion of the carriage. A cam-operated microswitch then changes over, stopping this motor and starting the carriage motor which remains energized until the carriage operates a limit switch. The motor is then de-energized and the relay system re-set.

To avoid stopping the motor suddenly the carriage runs forward until the pinion leaves the end of the rack.

A second group of relays permits the scaler to count only when the carriage is actually traversing the sensitive area, so avoiding the spurious pulses generated during starting and stopping.

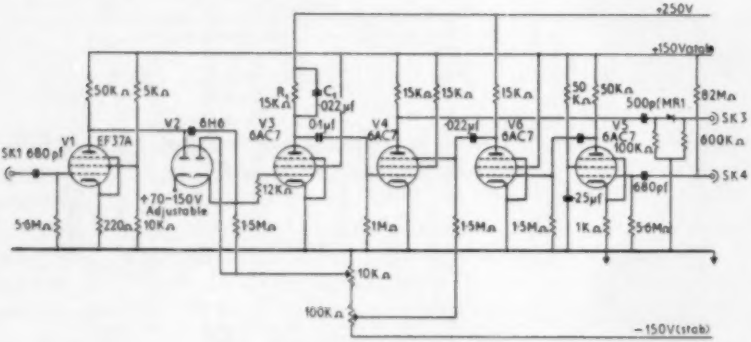

FIG. 2. Outline of amplifier circuit.

Fig. 2 is an outline of the electronic circuit. Details of power supply, stabilization, and decoupling have been omitted for the sake of clarity. Extensive decoupling was required to prevent erratic counting. The photomultiplier, type 931-A, is conventionally connected and normally operated at 720 volts E.H.T. and 680 K ohms anode load. The photomultiplier anode is connected to SK1, and the photocell (20 CV) anode to SK4 by flexible co-axial cables; the photocell cathode is connected direct to earth.

Negative-going pulses from the photomultiplier are fed to the discriminator V1, V2a. Pulses large enough to make V1 anode positive to the pre-set discriminator voltage on V2a cathode are passed to V3 grid, which is normally cut off. V3 therefore only conducts when the scanning spot falls on the background. The discriminator can be set to any level between 70 and 150 volts by a potentiometer.

The negative-going pulse at V3 anode is given an extended trailing edge by C1 and R1 to compensate for the shortening effect of the discriminator. This signal is used to cut off V4 at the control grid while the positive spike from the second photocell and amplifier is present on the suppressor grid. Pulses only appear at V4 anode when there are no pulses at the first grid, i.e. when the spot is on the leaf.

Pulses from V4 anode are shaped by MR1 and its associated network before being passed to the scaler.

PERFORMANCE

The instrument was routinely calibrated against rectangles of black paper between 25 and 600 cm.^{2} Sometimes single test pieces were used, at others the area was subdivided. Calibrations using leaves were more tedious, but agreed closely with the routine calibration. White paper lowered the counts by 15 to 20 per cent. The graph relating discriminator voltage to the number

of counts per cm.² had a plateau at least 35 volts long with a slope approximately proportional to the perimeter of the test area. A square test piece of 10 cm. side gave a slope of 3·8 counts/10 volts, the working range being 70 volts. Above the plateau the number of counts rises steeply as the darker

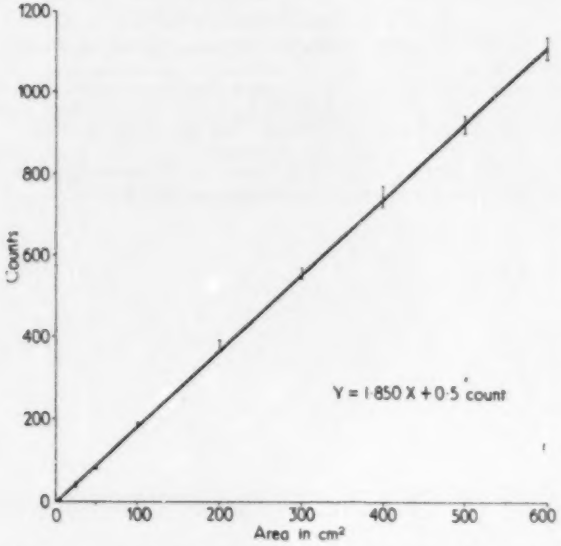

FIG. 3. Calibration curve using black card standards. The confidence limits ($p = 0{\cdot}05$) shown are for a single determination.

areas of the background begin to be counted. Normally the discriminator was set 10–20 volts below this level: this was convenient and gave good linearity. Dirtying of the trays and sensitivity drifts were compensated for by lowering the threshold.

By measurement, the distance between lines was 1·06 cm. and the distance between elements was 1·03 cm. corresponding to 1·83 pulses/cm.² Calibrations against card standards gave from 1·78 to 1·96 counts/cm.², and against leaves whose area was measured by the punch method (Watson and Watson, 1953) $1{\cdot}830 \pm 0{\cdot}076$ counts/cm.² ($p = 0{\cdot}05$).

For areas between 25 and 500 cm². the linear regression of counts on actual area accounted for over 99·9 per cent. of the sums of squares. The standard error was also approximately proportional to the perimeter of the test pieces; a 100 cm.² square gave a standard error of 3·3 counts for a single determination. Duplicate determinations had a root-mean-square difference of 1·9–4·5 per cent. of the mean, depending on the area and degree of subdivision.

The planimeter has now been in use for over a year without requiring servicing and has proved very simple to operate. In use, up to 48 m.² of

sugar-beet leaves (108 plants, 2,500 leaves) were measured in fewer than 5 hours. This performance suggests that instruments could usefully be designed to measure the areas of narrow and divided leaves and that portable instruments are practical possibilities.

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